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from Injury

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1961

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ON

RECOVERY OF CELLS FROM INJURY

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Introduction

'he recovery of cells from injury is one the basic problems in biological re- rch. The difficulties encountered in lying the processes that led to recovery 'e been great, and progress in this field not been fast. Consequently, it was ught that this might make a good topic a discussion where different points of v are brought out. This is especially ortant in connection with effects of iation on living cells where the recovery n assault is one of the basic problems connection with radiation exposure.

)n the basis of these ideas a sympo- n was organized by a committee from ; laboratory. Chairman was R. F. Kim- l with the following as his co-chairmen: [. Adler, N. G. Anderson, C. C. Congdon, D. Novelli, and Sheldon Wolff. The discussions brought out many interesting points in regard to the work which is going on in different laboratories with approaches from biochemical, biological and biophysical points of view. The discussions were taken down and are reproduced in essential parts in these proceedings.

This conference, as well as the previous conferences, was sponsored by the Biology Division of Oak Ridge National Laboratory in cooperation with the Division of Biology and Medicine of the Atomic Energy Commission.

The organizing committee was not only responsible for the setting up of the symposium, but also gave important contributions in editing the chapters for this volume.

Previous symposia in this series are:

1948—Radiation Genetics

1949—Radiation Microbiology and Biochemistry

1950—Biochemistry of Nucleic Acids

1951—Physiological Effects of Radiation at the Cellular Level

1952—Some Aspects of Microbial Metabolism

1953—Effects of Radiation and Other Deleterious Agents on Embryonic Development

1954—Genetic Recombination

1955—Structure of Enzymes and Proteins

1956—Biocolloids

1957—Antibodies: Their Production and Mechanism of Action

1958—Genetic Approaches to Somatic Cell Variations

1959—Enzyme Reaction Mechanisms

1960—Mammalian Genetics and Reproduction

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odification of Radiation-Induced Electron Spin esonances in Dry Materials

J. S. KIRBY-SMITH AND M. L. RANDOLPH
Biology Division, Oak Ridge National Laboratory,[1] *Oak Ridge, Tennessee*

The widespread occurrence of free radils in chemical, biological and radiobiogical processes is well established. Evince comes from classical chemical meths, from magnetic susceptibility studies of ramagnetic systems, and finally through e application of electron spin resonance SR) techniques.[2] This last approach, oreover, indicates the approximate numrs of radicals present. In some cases mething of the nature or structure of ecific types of free radicals is revealed analysis of ESR patterns. Therefore is field has rapidly expanded in the past w years and continues to proliferate into most all fields of biology and radiobioly. Although the actual involvement and portance of free radical mechanisms in diation action remains largely inferenl, the circumstantial evidence built up ESR (as well as by the more convennal chemical approaches) is now so rong that most of us agree that there is basic role of radical mechanisms in diobiological processes. Some of the most rect evidence on the biological role of ee radicals has been obtained by Powers. ee these proceedings.)

We do not mean to imply that all biogical effects of radiation are derived rough free-radical mechanisms; we do lieve, however, that free radicals freently play a significant role in the chain events following the primary physical ocess. Assuming that this is indeed e case, studies of radiation-induced free dicals and their decay constitute a pernent introduction to the general subject this symposium, the recovery of cells om injury.

Turning to the phenomenological apoach to radiation-induced free radicals, studied through ESR in dry systems, a mber of striking facts are immediately evident. Among these are: (1) extremely long lifetimes of many radiation-induced resonances, (2) relatively large numbers of these centers produced per unit of energy expended, (3) complex kinetics implied by decay schemes and dose curves, (4) dependence of the numbers of centers observed on linear energy transfer (LET), and finally, (5) dramatic manner in which the magnitude of the resonances may be changed by various postirradiation treatments. Although this paper is mainly concerned with this last characterization of free radicals, at least a minor review of the other experimental findings seems desirable.

Lifetimes and decay of resonances. One of the most striking characteristics of radiation-induced free radicals in dry systems, and one which makes postirradiation modification studies experimentally feasible and attractive, is the extremely long lifetimes of these entities. In contrast to molecules in excited states, many free radicals are intrinsically stable configurations and usually decay or are destroyed only through collision or recombination. Even in the liquid state, where rapid diffusion occurs, the effective lifetimes of some free radicals are long compared to those of excited, energetic and highly reactive electronic or vibrational states of molecules; in the gels and colloidial structures of biological systems they are undoubtedly even longer. In dry solids, radicals are found whose lifetimes may be days, weeks, months, or even years. This expanded time scale in solids permits simple, direct quantitative studies of parameters modifying the frequency and nature of primary resonances.

---

[1] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

[2] Discussion of specific techniques and details of the experimental methods of ESR are ignored in this presentation. Such material is well covered in volumes by Ingram ('58) and Blois *et al.* ('61).

Although relatively slow, the decay of radiation-induced resonances observed in dry materials is complex and cannot in general be expressed by a single exponential relation. In some instances decay studies indicate the initial formation of two or more species which disappear at different rates; in others, evidence for conversion of one species into another is experimentally observed as in irradiated lyophilized bacteria (Lion *et al.*, '61) and in the work of Powers ('60, '61) and his coworkers on spores. In some cases we have found decay schemes that depend upon the magnitude of the applied radiation dose, i.e., on the concentration of radicals initially present (fig. 1). Studies of these decay phenomena afford a promising approach (1) to an understanding of the mechanisms of formation and recombination of free radicals and (2) under conditions of high resolution, to specific changes in their chemical structures.

Numbers of centers produced. Direct determinations by Zimmer ('59), Zimmer, Ehrenberg, and Ehrenberg ('57), Box and Freund ('59), and Randolph ('61) and others have shown conclusively that radical formation in dry materials by ionizing radiation is an extremely probable event. Energy expended per radical pair produced in representative amino acids and proteins ranges from 15 to 1000 ev, approximating in many instances the energy (W) expended in the production of an ion pair. Radicals are thus produced in sufficient numbers to account for many of the secondary effects of radiation. If radical processes are to be ignored as a trivial side effect of radiation unrelated to radiation damage, this must be justified on other grounds.

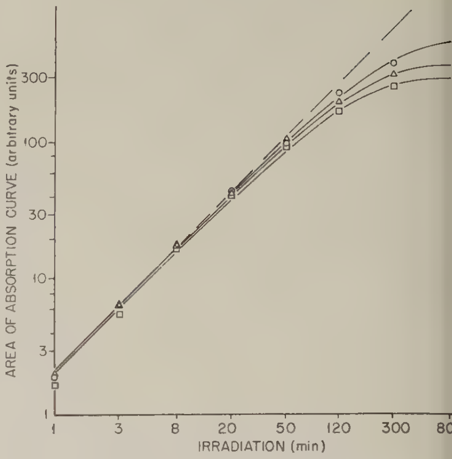

Fig. 1 Concentration of resonances as a f
tion of dose and time. At high doses note
saturation effect and more rapid decay tha
low doses. ○, day of irradiation; △, 2 days a
irradiation; □, 13 days after irradiation. Gl
mic acid irradiated at 80 kr/min.

LET dependence. Studies by Ki
Smith and Randolph ('60), and by
('60) show an inverse dependence of
production of resonant centers on L
(table 1). This decrease in the efficie
of radical formation as LET is increa
is very marked for all systems studi
amino acids, proteins, and polysacc
rides. Conger and Curtis ('61) have fo
fast neutrons are ineffective in induc
resonances in dry seeds. High concen
tions of radicals formed along and parti
larly at the ends of densely ionizing tra
may be expected to result in immediate
combination of primary radicals and t
lower the signals observed after irrad
tion. Local heating, analogous to "ther
spikes" at the end of the tracks, sho
also facilitate recombination process
Hochanadel ('61) in studies of the rad
ysis of nitrate crystals has found evide
supporting a "thermal spike" effect.

TABLE 1

Relative production of resonances by different radiations

Irradiated material	Radiation			
	γ (Co)	X (250 kvp)	N (14 Mev)	α (Po)
Glycine	1	—	0.7	0.3
Chondroitin sulfate	1	0.6	0.45	0.05
Gelatin	1	0.9	0.8	0.1

Postirradiation modification of magnetic onance signals in solid chemical sys- s. The magnitude of the resonance nals observed in all solid chemical and chemical systems (as well as in the dry logical materials to be considered later) drastically reduced by postirradiation posure to either ultraviolet (UV) radi- on or to heat. Zeldes and Livingston 9) as well as Alger, Anderson, and Webb 9) have clearly demonstrated the action visible and UV light in modifying the onances observed in alcohols, ketones d other organic compounds irradiated low temperature.

In polycrystalline glycine our qualitative sults indicate an approximately exponen- l decrease of resonance centers with in- asing dose of UV radiation. In this experiment the material was exposed in the form of a finely ground powder suspended in an inert liquid (hexane) that transmits UV. Similar results have been found for postirradiation heat treatment. Experiments performed on single crystals of glycine lead to comparable results and have the added advantage that changes in UV absorption spectra may be compared with the spin resonance data. Kondo ('59) has shown that exposure of irradiated glycine crystals to heat results in considerable change in the UV and visible absorption spectra. Our more complete data for the effects of both heat and UV radiation of 2537 Å are given in figures 2–4. These curves show also that disappearance of the radicals is not correlated with a shift

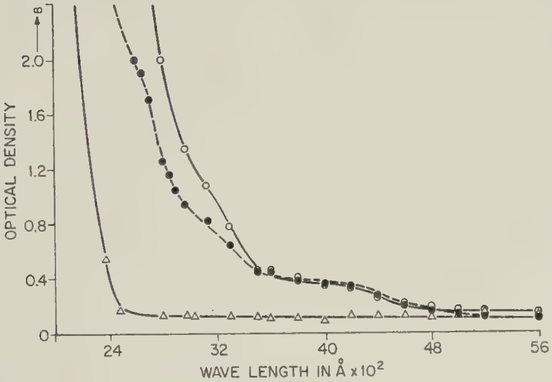

Fig. 2 Modification of absorption spectra of single glycine crystals induced by various radiations and posttreatments. ○, X-irradiated plus UV treatment; ●, X-irradiated; △, unirradiated.

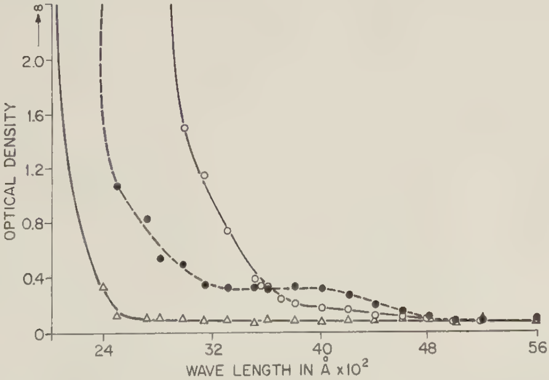

Fig. 3 Modification of absorption spectra of single glycine crystals induced by various radiations and posttreatments. ○, X-irradiated plus heat treatment; ●, X-irradiated; △, unirradiated.

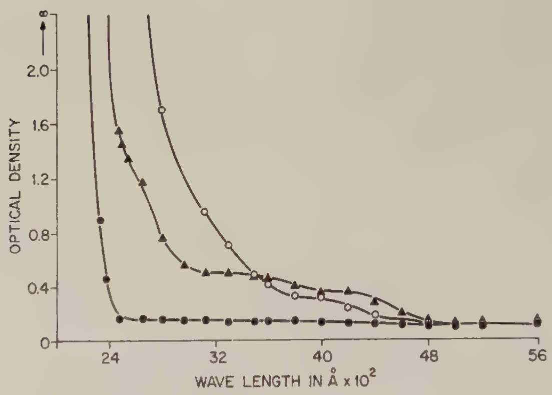

Fig. 4 Modification of absorption spectra of single glycine crystals induced by various radiations and posttreatments. ○, X-irradiated plus UV plus heat treatment; ●, unirradiated; ▲, X-irradiated.

in the UV absorption spectrum back to its initial shape in unirradiated crystals.

These facts emphasize one of the most fundamental questions concerning the decay or other modifications of the free radical signals, namely, the mechanisms and end products involved in these processes. Before speculating on this matter or even attempting to give an answer, some essential and basic aspects of free-radical production often ignored if not overlooked must be mentioned. Radicals are not produced singly; in all cases one can imagine they must occur in pairs. Although, as Livingston ('59) has pointed out, the frequent absence of two distinguishable resonance signals is puzzling, two types of radicals must exist. Two possible explanations for this condition are (1) an extremely broad resonance associated with one of the species or (2) a resonance falling at some markedly different portion of the magnetic spectrum. If one member of the pair is a hydrogen atom, owing to the high mobility of H atoms, the formation of molecular hydrogen, rather than recombination of primary radical pairs, may take place. Another possibility is a very rapid transformation of primary radicals to an undetectable species as indicated in (1) or (2) during and immediately after irradiation before the first ESR measurement.

Recognizing that radicals are produced in pairs after irradiation, one can understand in a general way how heat treatment so drastically reduces the radi
concentrations. Increased thermal mot
should result in a higher probability
collision and recombination of the prim
pairs. After the primary rupture of
original molecule, that part of the init
signal not removed by heat treatm
could conceivably originate from a radi
whose partner, perhaps a hydrogen ato
has moved too far away in the lattice
further interaction. Our results for he
treated glycine also indicate that the re
nances decay to a plateau, below wh
continued heating is ineffective.

The action of UV radiation in reduc
the resonance signal is somewhat m
difficult to rationalize, particularly in s
tems such as glycine where UV alone p
duces few if any free radicals. The
parent exponential reduction in numb
of primary radicals with posttreatm
dose of UV (fig. 5) does however indic
"one-hit" kinetics; from this standpoin
mechanism involving the interaction
an UV quantum with a radical and sub
quent recombination of this excited str
ture with its partner might follow.

One further point concerning radic
must also be kept in mind, i.e., the la
amount of stored energy a radical pair p
sesses—the exact value depending up
which covalent bonds have been brok
On recombination, this energy must so
how be dissipated, and in this proces
considerable amount of localized chem
or biological damage may be produc

is, in some systems, the disappear-
e of resonances may be associated with
logical damage rather than "protection."
will return to this point later in the
cussions of modification of electron spin
onances in some representative dry bio-
ical systems.

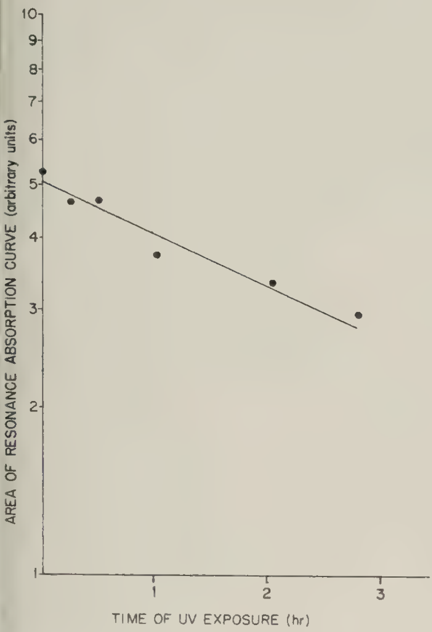

Fig. 5 Effect of UV posttreatment on X-ray-
duced resonances in glycine.

Studies of the structure of the absorption envelopes provide direct evidence concerning the structure of radicals and the identification of different radiation produced species, as well as evidence for the decay from one species to another. This is well illustrated by studies of irradiated glycine. Resonance spectra of irradiated polycrystalline and single crystals of glycine are shown in figure 6. The original interpretation (Gordy *et al.*, '55) of the polycrystalline pattern with its distinctive triplet is that it arises from a (CH_2) radical, although the presence of the wings does raise some doubts. More recent and very beautiful spectra of single crystals under high resolution have been obtained by Ghosh and Whiffen ('60). Further analysis of the spectra by these investigators indicates that irradiation of glycine by γ-rays leads to the predominant formation of the radical (NH_2). These radicals disappear after several days at room temperature with the permanent appearance of a species believed to be $(^{+}H_3N\dot{C}H\,CO_2^{-})$. After prolonged heating this radical also disappears to leave a third but yet unidentified species.

One further and very obvious manner of modifying or eliminating free-radical reso-

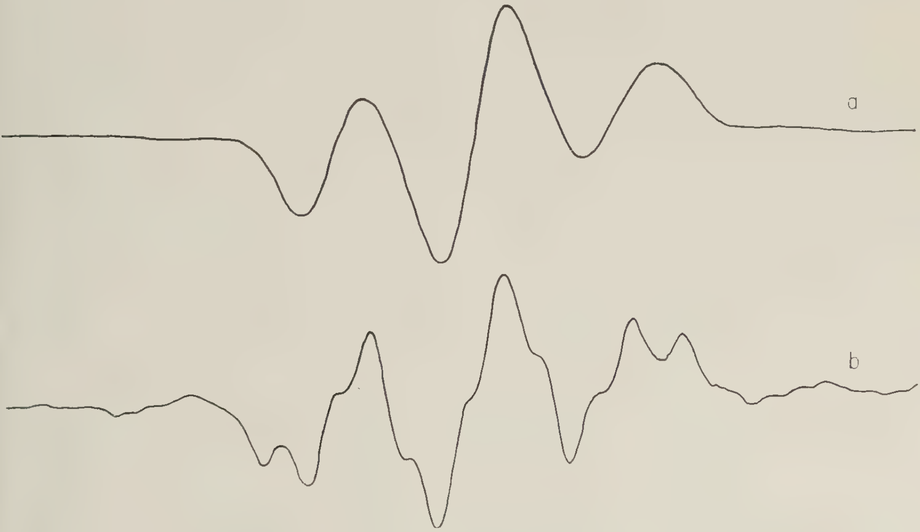

Fig. 6 Resonance spectra of irradiated glycine: a, Polycrystalline; b, single crystal oriented with its b axis perpendicular to the magnetic field and its c axis at an angle of 120° with the direction of the field.

nances, which has at least been implied if not mentioned, is exposure to moisture. When irradiated DNA, proteins, and other dry chemical systems, as well as dry pollen and bacteria, are placed in water, or even in many instances merely exposed to conditions of high humidity, the resonances rapidly decay. In view of the evidence and mechanism postulated, this effect is possibly a result of increased mobility of radicals under these wet conditions which results in more rapid recombination, rather than a direct reaction of radicals with water molecules.

Postirradiation modifications in dry biological systems. Having indicated briefly the actual phenomena observed and some of the physico-chemical processes which may operate in the postirradiation modification of resonances in solid chemical systems, it is of interest to consider a few representative dry biological systems. Before discussing any specific system we are compelled to emphasize again that insofar as biological effects are concerned we have no direct incontrovertible proof of any definite causal relationships between free radical concentrations and biological damage; in fact, in the first example to be considered, dry *Tradescantia* pollen, the biological damage may be correlated with the disappearance of the free-radical signals.

Tradescantia dry pollen. General exploratory studies on radiation-induced ESR signals in *Tradescantia* pollen have led to very similar experimental results as those outlined for the amino acids. Heat, UV, and moisture each cause a drastic reduction—or in the case of water a complete disappearance—of the resonance signals.

Tradescantia pollen is an ideal material for many biological purposes, particularly for studying chromosomal effects of almost all types of radiations, and is particularly suitable for studies of combined treatments with different agents. Some years ago Miss Roberta Lovelace at the ORNL Biology Division investigated chromosomal breakage by combined γ-rays and UV radiations with inconsistent results, and the work was dropped. The marked drop in spin resonance caused by UV posttreatments prompted a second look at the possible modification of X-ray-induced chromosomal damage by UV radiati (Kirby-Smith *et al.*, '61). In a comprehe sive series of experiments we determin the general parameters which had led the previous variations in results report by Miss Lovelace. We expected a decrea in aberrations in accord with the ESR dat but in every experiment performed, po irradiation treatment with UV led to a increase, rather than a decrease, in chr mosomal aberrations. This synergism also observed for pretreatment with U radiation. If the effect existed only f posttreatments, we might, by invoking th energy released on radical recombinatio be able to "explain" this phenomenon i such terms. A possibility remains th pretreatment with UV produces radica which in recombination with those i duced by subsequent X ray treatmen result in biological damage, i.e., chrom somal aberrations. Further consideration of this synergistic effect lead us away fro the general subject of this presentation An alternative explanation which does n directly involve free-radical mechanism leading to chromosomal breakage no appears more probable. This explanatio based on interference with rejoining mech anisms, is developed in detail by Sheldo Wolff and R. F. Kimball elsewhere in th volume.

Seed germination. We turn next t work on the relation of ESR signals an germination in lettuce seeds (Randolp and Haber, '61). The combination of bio logical (germination) and physical (ESR sensitivities of this material are fortunat because, unlike many correlations of ES and biological effects, germination can oc cur after doses that produce measurabl free-radical concentrations. Thus conclu sions drawn from data on this material ar free from the objection that ESR studie involve doses far in excess of those bio logically important.

Free radicals are produced in these dr X-irradiated seeds on the absorption c about 300 ev per resonance at low dose with the concentration approaching a satu ration limit of roughly 10^{18} spins/gm a doses of 20 Megarads. Most of these reso nances are produced in the embryo, a judged from mechanically separated see

ts, and their decay rate is increased by
isture or heating.

Preliminary parallel germination and
R studies after soaking these seeds in
-aminoethylisothiourea dihydrobromide
ET) or water, drying, and X irradiating
owed: (1) maximum germination at an
T concentration of $\approx 10^{-4}\%$ 10^{-3} M;
d (2) the ESR dependence on AET con-
ntration and time shown in figure 7. Dry
adiated AET exhibits a quite specific ESR pattern which was not seen in these AET-treated seeds. Hence the higher initial concentration of radicals seen with the AET treatments cannot be merely the sum of unrelated signals from seed and dry AET but might be from seed and AET derivatives. However, from rough-weight measurements, if such AET derivatives account for the enhanced initial ESR signals, the derivatives must be about 50 times as sensitive to radiation as any other material we have studied. Furthermore, crossing of the decay curves at about 100 hours after irradiation also excludes the possibility of simple addition of two unrelated species of resonances, but does not exclude the possibility of a rapidly decaying AET derivative system and a seed system with postirradiation decay accelerated by AET. These considerations suggest modification of the radiation-induced free radicals in seeds by a biologically significant AET treatment. Virtually no germination was found in either water controls or any AET-treated seeds stored for 12 days after irradiation to permit decay of free radicals in the dry state, a finding qualitatively similar to other investigators' experience with irradiated seeds. These AET and

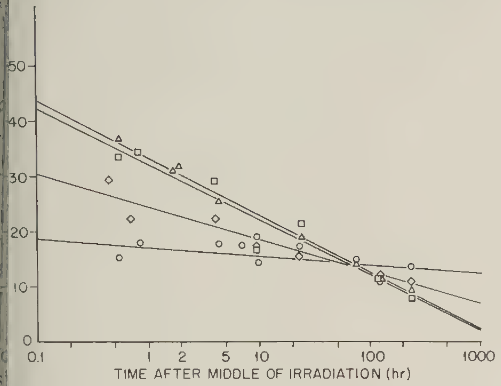

Fig. 7 Decay of ESR signal induced in lettuce
ds as a function of AET concentration and
e. ○, H_2O; ◊, 10^{-3} *M* AET; □, 10^{-2} *M* AET;
10^{-1} *M* AET.

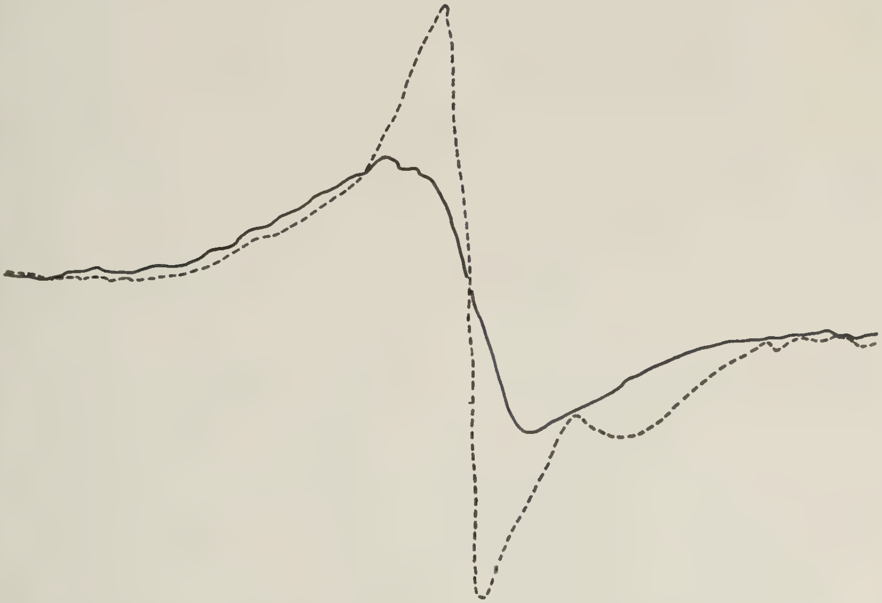

Fig. 8 Change in radiation-induced ESR pattern in lyophilized *E. coli.* —— Initial signal; - - - - signal after heat treatment.

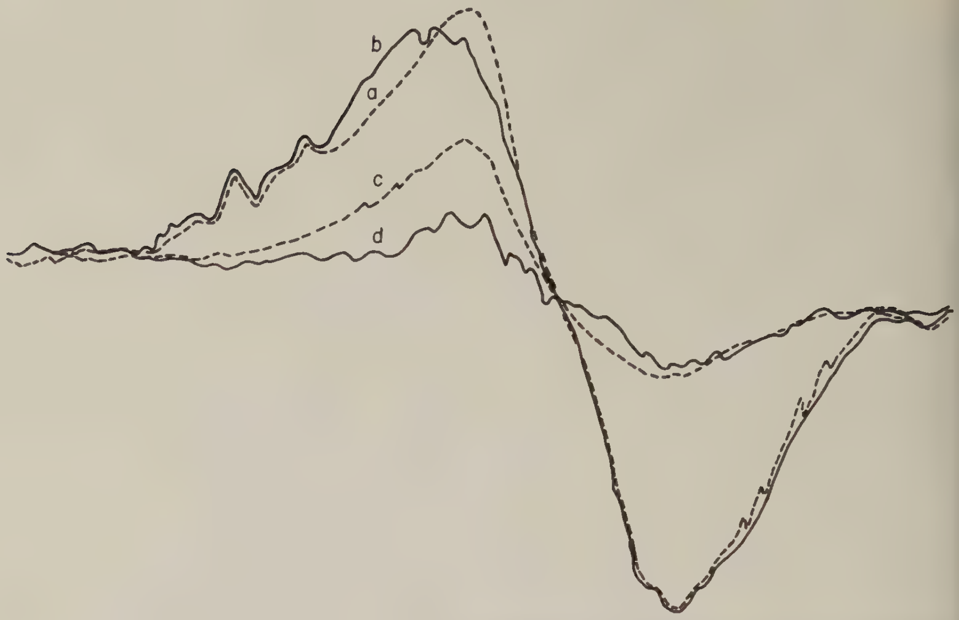

Fig. 9 Suppression by various additives of naturally occurring ESR signal in lyophilized *E. coli* cells exposed to oxygen. a. no additive; b. cysteine; c. thiourea; d. NaI.

storage results suggest that biological damage in this material might better be related to number of resonances decaying in the dry state than to the number of resonances measured at any particular time.

Dry bacteria. We (Lion, Kirby-Smith and Randolph, '61) recently observed some interesting and provocative relationships between free radicals and viability in both irradiated and nonirradiated lyophilized *E. coli* cells.

Dried bacteria irradiated with 750 kr of X rays in the absence of oxygen showed a broad, weak, and double-peaked signal which became higher and sharper after heat treatment (fig. 8) and rapidly decayed on exposure to oxygen. This change in pattern after heating the irradiated material suggests a heat-induced transition from one radical species to another, and a fundamental difference in radiation-induced radicals and those occurring after lyophilization alone which showed little if any change with similar heat treatment. This signal increased to a maximum after several hours and remained constant for at least six weeks.

When *E. coli* cells are lyophilized in distilled water and then exposed to small amounts of air or oxygen, a very rap
loss of viability of the cells may be o
served, while no such effect is produce
when the cells are kept under vacuu
Dried *E. coli* cells may also be protecte
against this lethal effect of oxygen by thi
urea or more effectively by sodium iodid
but cysteine is completely ineffective
this respect. In a typical experiment (Li
and Bergman, '61) the viability of dri
cells after exposure to oxygen was foun
to be: control 0.05%; in presence of cy
teine 0.001%; in presence of thiour
42%; in presence of NaI 80%.

Lyophilized bacteria kept *in vac*
showed no perceptible spin resonance si
nal. In the presence of oxygen a relative
strong signal was observed 30 minut
after exposure. Contrary to experimen
on nonviable lyophilized tissues of anim
origin by Miyagawa *et al.* ('58) the sign
also remained stable even after re-evacu
tion and removal of oxygen. In expe
ments where the additives, cysteine, N
or thiourea were used, a definite correl
tion between the decreased free-radic
signals and increase in viability was o
served (fig. 9). From these results t
bacterial cell appears to be a promisi

erial for further ESR studies of the
of naturally occurring, free-radical
hanisms in a living system.

CONCLUSIONS

he modification of spin resonances in
condensed chemical systems and dry
semi-dry biological materials follow
tical or closely parallel mechanisms.
s, we may expect further studies of
ation-induced radicals in chemical or
hemical systems, studies which in
nselves are a fascinating branch of
ation chemistry and solid state physics,
e of continued application to the radio-
ogy of semi-dry organisms.

ooking toward the future role of ESR,
extension of this approach to radiation
lies in wet, typically metabolizing sys-
s holds great promise. Improved tech-
ues now permit studies of enzyme
chanisms to be made, and there is good
son to believe that the kinetics of
rter-lived radiation-induced resonances
vet, typically metabolizing living mate-
s can ultimately be elucidated by ESR
hods.

OPEN DISCUSSION

EINMETS[3] : I am confused about this
energy—up to 2000 ev—required for
cal formation. Is this considered a
gle-step energy required for a radical
nation, or is it considered the average
radical?

IRBY-SMITH: You are confusing the
rgy expended per radical formed (30–
0 ev), a quantity somewhat analogous
V, with the energy required to produce
dical. This latter amount is certainly
more than the ionization energy, i.e.,
roximately 10 to 15 ev, and probably
ess.

UTCHINSON[4] : To what extent do meas-
ments of this kind actually measure
total number of radicals formed?

f one gets a clear pattern in the usual
ivative plot, this can frequently be as-
ed to a single radical. Superimposed
this single radical pattern could con-
ably be a very broad pattern due to
superposition of a great many differ-
radicals.

s the figure of 15 ev per free radical
nd for glycine due to the fact that in glycine a single radical is always formed; whereas in more complicated structures a good deal of the energy may go into forming a great many different kinds of radicals?

Kirby-Smith: The measured number of electron volts per radical is an upper limit due to the possibility of broad undetectable resonances. Superposed narrow resonances would be detected. In the case of glycine the low value of ev per radical reflects probably the stability of the radicals produced rather than indicating the existence of a single species.

Hutchinson: Do you mind if I pin you down a little more on this? You mean that, in the normal measurements of this type, there are probably radicals that are not detected?

Kirby-Smith: Yes, there may be radicals having broad resonances which are undetectable. In addition some recombinations may have occurred before measurement.

Failla[5] : In the case where you have 2000 ev, is the decay more rapid than in the case where you have 15 ev? In other words, is the value large because there has been decay in the meantime?

Randolph: We have not systematically studied the question you have asked, Dr. Failla. In some cases I think this is true; but we do not have enough data to generalize now.

Failla: That would happen in determining the value of W (the energy lost by an ionizing particle per ion pair produced) if ions are allowed to recombine a larger value results.

Wolff[6] : Perhaps we should mention the alternative explanation that Dr. Kirby-Smith alluded to, concerning the synergism in the action of UV and X rays. That is, the data are consistent with the concept that we are knocking out the rejoining processes that go on. With ionizing radiation we have calculated that most of the breaks restitute.

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[3] F. Heinmets, Quartermaster Research and Engineering Center, Natick.

[4] F. Hutchinson, Yale University.

[5] G. Failla, Argonne National Laboratory.

[6] S. Wolff, Oak Ridge National Laboratory.

Many more primary breaks are put in than show up as final aberrations. These data of Kirby-Smith and Nicoletti are consistent with the point of view that what we are doing with the UV is preventing the restitution so that the breaks that would ordinarily disappear now take part in rejoinings that give rise to visible aberrations.

Kimball[7] : In Paramecium, we have found a decrease in the amount of recessive lethal mutation with combined UV and X-ray treatment. The combined radiations produce less mutation than the X rays alone. We have suggested (Kimball, Gaither, and Wilson, 1959, Radiation Research 10: 490) that UV delays chromosome duplication, thereby allowing more time for recovery of the X ray-induced mutations. In support of this, mutagenic doses of UV delay division very considerably but mutagenic doses of X rays do not.

von Borstel[8] : As a third alternative, Dr. Löbbecke and I have found fairly strict additivity for UV and X rays in the particular system that we are using.

To frame a general conclusion, it is possible that the recovery system may be more sensitive to UV radiation than to X radiation in certain systems, and in other cases the recovery system is superfluous.

Kirby-Smith: In our case we are working with a very dry slowly metabolizing system. Significant differences in the response of wet and dry biological systems to radicals certainly exist.

Gray[9] : Is there any correlation between the spin pictures as a function of moisture content of the spore and the biological effects?

Kirby-Smith: The only correlation in the spin picture is the general one that, if we irradiated the pollen grains dry and expose them to high humidity, the resonance signal drops very rapidly in the order of a few minutes.

Biologically, the sensitivity of the pollen grains to UV is drastically reduced at high humidity.

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[7] R. F. Kimball, Oak Ridge National Labora

[8] R. C. von Borstel, Oak Ridge National L ratory.

[9] L. H. Gray, Mt. Vernon Hospital, Northw

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eversibility of X Irradiation-Induced Effects in ry Biological Systems[1]

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There are two general classes of revers-
e events or states that must be distin-
ished. There are radiation-induced bio-
ical events that may be reversible; and
re are reversible radiation-induced
nts that lead to these reversible bio-
ical events or, perhaps, to irreversible
es. We should expect that the processes
reversal will be different in the two gen-
al categories. For instance, the repair of
broken chromosome should depend upon
tors and show characteristics that are
ferent from those exhibited by reversal
removal of the radiation-induced chem-
l species that precede in time and would
d to the chromosome break if allowed to
nain in the cell. Both are examples of
ersible radiation effects. One is a "bio-
emical" or "enzymatic" type of revers-
lity; the other is chemical or physico-
emical. The purpose of this paper is to
mine in bacterial spores radiation ef-
ts that probably are physico-chemical
nature, with the particular purpose of
parating the reversible from the irrevers-
e (or those that are reversed with greater
ficulty), and of noting the effect on the
asured response of the cell to the
rying degrees of reversibility that we can
serve.

The end-points we shall examine are
logical in nature—the ability of an ir-
liated spore to germinate and develop
o a cell that can then go through ap-
oximately 10 fissions. The radiation ef-
ts that we shall manipulate are physico-
emical, not biological, in nature. It is
portant to appreciate that, in this way,
may gain an understanding of the rela-
nship between physical and chemical ef-
ts of radiation in cells and the biological
nsequences of these effects. This under-
nding is impossible, or nearly so, if one
amines only one or the other.

The system that we use is particularly suitable for this kind of analysis, for in this almost dry cell no metabolic activity occurs before, during, or after irradiation. The varying biological effects we see can certainly be ascribed to antecedent physical and chemical effects; and the use of the biological end-point of colony formation measures directly, in one respect, the biological importance of these physico-chemical events. We should expect to recognize precise behavior on the part of the reversal processes if we can isolate them for measurement. We should expect to be able to predict the consequences of the rules that we recognize, even though the interrelations among them may be complex.

This paper will present the results of an examination of the radiation response in terms of subunits that are experimentally recognizable and measurable. It will demonstrate their characteristics and behavior and will show that understanding of these elements of the over-all radiation response must be realized before an understanding of complex responses to radiation is possible.

THE EXPERIMENTAL BASES

Before considering the characteristics of the various portions of the radiation response in the bacterial spore, we review briefly the evidence for dividing the response into recognizable categories. It is summarized in the diagram we call the "radiation sensitivity profile" (Powers and Kaleta, '60) reproduced as figure 1.

In spores prepared and irradiated in our standard manner (Powers, Ehret and Bannon, '57; Kaleta and Powers, '58; Webb, Ehret and Powers, '58) at room temperature and at relatively high dose rates

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[1] This work was performed under the auspices of the United States Atomic Energy Commission.

(about 16 kr/min.) maximal sensitivity is observed when they are exposed to X rays in the presence of O_2. When the atmosphere is N_2, and the irradiated spores are exposed to O_2 immediately thereafter, radiation sensitivity drops, indicating that O_2 is responsible for damage by interaction with short-lived radiation-induced species in this cell. This is the Class II part of the over-all radiation response, and in the experimental circumstances being described, it accounts for about 25% of the entire response.

When, after anoxic irradiation, the spores are heat-treated at 80°C. for 15 minutes (Webb, Powers and Ehret, '60), or exposed to NO (Powers, Webb and Kaleta, '60), or exposed to H_2S (Powers and Kaleta, '60) prior to exposure to O_2, a further drop in radiation sensitivity is observed. Exposure to O_2 prior to these treatments prevents this effect. Because of these facts, and because of evidence from electron spin paramagnetic spectroscopy (EPR) data (Ehret, Smaller, Powers and Webb, '60), the reduction is ascribed to the removal of free radicals which, in combination with O_2, become rapidly harmful to the cell if allowed to remain. We term this portion of the response Class III. Note that the O_2 effect in this cell consists of two parts, one (Class II) that occurs quickly, and one (Class III) that can occur at times considerably after irradiation. Class III constitutes about 40% of the maximal effect seen.

The remaining portion of the response (Class I) is that which is entirely independent of O_2. It constitutes about 40% of the over-all effect. The presence of H_2S during anoxic irradiation results in the reduction of sensitivity to a level appreciably below that seen after postirradiation thermal annealment, or NO treatment, indicating to us the presence of a transient, radiation-induced state or states (Class Ib) that becomes harmful unless removed or nullified in some way by H_2S (Powers and Kaleta, '60).

The general expression that we hav used to describe the survival of thes spores after irradiation under these cond tions, is

$$S = 1 - \left[1 - \exp(-kD)\right]^{n} \quad (1$$

where *S* is the fraction surviving, *D* is th dose, *n* is a small positive constant usuall greater than 1.00, and *k* is a constant tha takes different values under different e perimental conditions. The constant *k* our measure of radiation sensitivity an is called the inactivation constant. In a our papers to date we have used this e pression in a purely formal way, and hav carefully stated that no interpretation were to be put on any of the constant Under the conditions stated in figure 1, w see, however, that the measure of radiatio sensitivity, *k*, can really be written as

$$k = k_{I} + k_{II} + k_{III}$$

that is, the total is the sum of the contribu tions to the slopes of Class I, Class II an Class III events.

In practice, of course, we do not measur each of these separately, but rather inf their magnitudes by differences. Thus, i practice,

$$k_{III} = k_{N_2} - k_{NO}$$

k_{N_2} being the sensitivity of the spore irradiated in N_2 and exposed immed ately to O_2, and k_{NO} is the sensitivit (inactivation constant) observed aft maximal postirradiation reversal of th anoxic irradiation effect with nitric oxid When irradiation occurs in the presence O_2, the quantity added in terms of th measurements actually made is

$$k_{II} = k_{O_2} - k_{N_2}$$

i.e., the maximum response minus the fre radical (Class III) and oxygen-independen (Class I) components. Thus the gener expression in terms of the measuremen when irradiation takes place in the pre ence of O_2 is shown in equation (2).

$$S = 1 - \left[1 - \exp\left(- \}(k_{NO}) + (k_{O_2} - k_{N_2}) + (k_{N_2} - k_{O_2})\{ \cdot D\right)\right]^{n} \quad (2$$

In this expression we see actual meanings in physical and chemical terms, at least for the *k*'s if not for *n*. k_I is the rate (kr^{-1}) of formation with increasing dos of oxygen-independent species that resu in loss of colony-forming ability in th

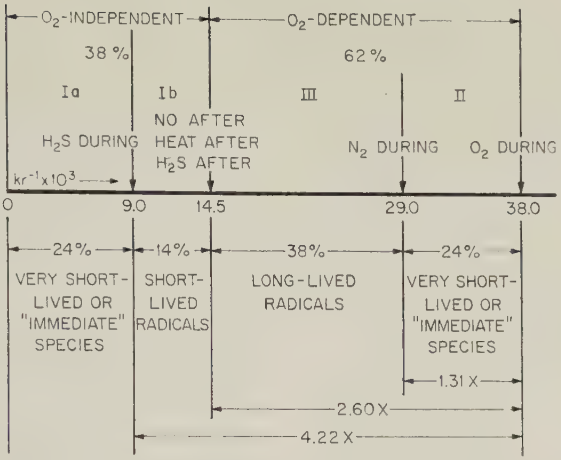


Fig. 1 The "radiation sensitivity profile" showing the relationships between the inactivation constants obtained when dry spores are irradiated with 50 kvp X rays at room temperature at about 16,000 r/min. The heavy horizontal line indicates the constant observed under the experimental conditions indicated (Powers and Kaleta, '60).

re. k_{III} is the rate of increase with dose ree radicals that with O_2 prevent colony nation, and k_{II} is the rate of increase of y short-lived species that are harmless ess O_2, and perhaps NO, are present at time of irradiation.[2]

efore this time, we have used the in- vation constant in a general way to id prejudicing our interpretation with ubstantiated notions of its meaning. henceforth, we shall think of the in- vation constant in these measurable sical and chemical terms. With this roach we can begin analysis of and ive at certain physical explanations for behavior of the irradiated bacterial re.

The short-lived O_2-independent species (Class I)

The part of the response that seems most rly "immediate" is that we call Class I, oxygen-independent portion. As noted ve, it can be subdivided on the basis of reduction in magnitude observed when S is present at the time of irradiation. have several working theories concerning this and other aspects of this portion of the radiation response, but they do not bear upon the question of reversibility of radiation effects in the sense that we wish to discuss them here. Therefore, no further description of this class will be made, except to note that, as demonstrated in figure 2, Class I is partly temperature-de-

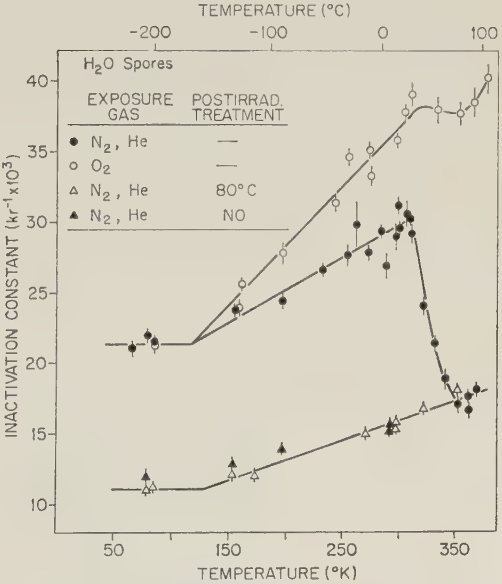


Fig. 2 The relationship between radiation sensitivity and temperature under the experimental conditions described in the figure (Powers, Webb and Kaleta, '60).

NO when present at the time of irradiation de- ases the net protective effect of NO observed en it is given after irradiation, indicating that re are two effects. One is the removal of free icals, and is protective; the other is a small ancing effect which may be the same as that O_2 (Powers, Webb and Kaleta, '60). A full cussion of this phenomenon is being deferred il a later time.

pendent. Since further developments in this paper depend upon the magnitude of Class I, we must be aware of this characteristic. It will remain in expression (2) since all constants in the equation depend upon it; but, other than its regular variation with temperature, it is considered in this paper to be independent, invariant, and irreversible. Actually it does vary in some circumstances, but not under the conditions used in these experiments.

The short-lived O_2-dependent species (Class II)

It is necessary for O_2 to be present at the time of irradiation for this component to be seen; we conclude, therefore, that the phenomenon of oxygen enhancement is due to species or events that in our time scale are short-lived or fast. Of course, they need not be immediate. For example, the lifetime of an excited singlet state might be 10^{-8} seconds, and the deciding harmful event could occur at least this long after energy absorption. Extension of this lifetime by a factor of 10^4 would lengthen it only to 10^{-4} seconds, a lifetime that is still very short with respect to our experimental techniques.

The explanation of the Class II effect may be of this nature. In fact, we have previously proposed such a mechanism as a possibility (Powers, Webb and Ehret, '60). There are several physical studies that support such a notion (e.g., Tsubomura, Mulliken, '60), and it is testable in the dry spore system. However, in this discussion we shall not further treat this Class II. Since the values of Class I and III do not depend upon an estimation of the value of this, for the present it is withdrawn from equation (2).

The free-radical component (class III)

It is this component of radiation injury that, lasting appreciable lengths of time, is most susceptible to reversing influences that affect survival of individual spores, and therefore, that affect the nature of the dose-survival relationship.

The evidence we have presented in the literature to date for the existence of this component, and the relationship between these radicals and oxygen in bringing about

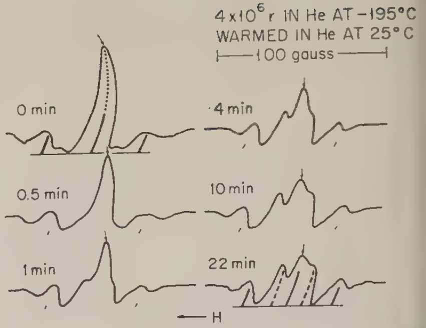


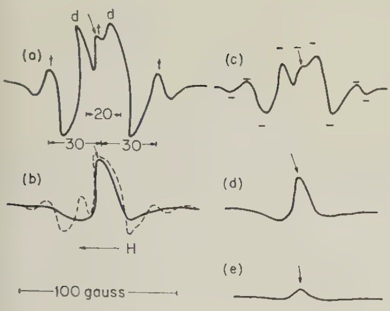
Fig. 3 The development of the EPR sig with time after irradiation. The two gene kinds of radicals (one indicated by the doub and the other by the triplet) are seen only af heating the irradiated spores at room temperatu after irradiation (Ehret, Smaller, Powers a Webb, '60).

radiation effects in the cell (Powers an Kaleta, '60; Webb, Powers and Ehret, '6 Powers, Webb and Kaleta, '60; Ehre Smaller, Powers and Webb, '60; Power Webb and Ehret, '60; Powers, Webb an Ehret, '59; Powers, Ehret and Smaller, '6 can be summarized as follows:

1. At room temperature the free radica have appreciable lifetimes (hours).
2. They effectively disappear biologicall after exposure to NO, H_2S, and other rad cal scavengers, and after heat treatmen
3. This disappearance is not possible spores exposed to oxygen after being radiated in nitrogen. Oxygen combin with the radicals to form toxic complexe
4. EPR studies indicate the presence two kinds of radicals after irradiation, o of them formed immediately and the oth developing slowly in time (fig. 3).
5. The EPR studies show that these ra icals can form oxygen complexes, as t biological studies indicated previous (point 3) (fig. 4).
6. They show also that heat, NO a H_2S affect radical content (point 2 f biological evidence), but perhaps in d ferent ways (discussed below).

Evidence for differences among the radicals

Several lines of evidence suggest th distinctions between or among the radica are possible. The apparent differences b tween their behavior judged biological and judged physically (Powers, '61) co stitute one reason. The postirradiatio



ig. 4 The behavior of the EPR signal ob-
ed in X-irradiated bacterial spores following
tments that lead to modification of biological
onse to irradiation. (a) The fully developed
cal signal. (b) After exposure to air, after
diation. (c) After thermal annealment—
C. for 15 minutes. (d) After exposure of (c)
ir. (e) After exposure to NO (Ehret, Smaller,
ers and Webb, '60).

atments of the spores with NO and with
t have the same effect on survival,
r-all sensitivity being reduced to half
anoxic sensitivity (fig. 2). However, the
ponse of these radicals to the two treat-
nts is different, according to EPR meas-
ments (fig. 4). Without changing the
lity of the signal, heat reduces concen-
tion to about half, in agreement with
biological results. NO, on the other
d, removes almost all evidence of the
nal. Since O_2 reacts with the radicals
t remain after annealment, two alterna-
s are presented. These radicals, which
unannealable, do not participate in the
logical damage, since their removal by
does not reduce biological effect. Or,
se radicals, after reaction with oxygen,
cause damage. Therefore, since they
react with NO, this must result in dam-
also. NO, then, has two actions—one
emove harmful radicals by reaction to
n the non-radical R_1ON species that is
mless; and two, to react with unan-
lable radicals to form the non-radical
cies R_2ON that is harmful. $R_1\cdot$ must
er in some way from $R_2\cdot$ (Powers, '61).
variations do not reside in the differ-
es between those radicals whose pres-
e is indicated by the doublet and those
the triplet signals, since both of these
affected in like manner by the experi-
tal procedures.

TABLE 1

Inactivation constants obtained after irradiation in O_2-free atmosphere, and then exposed to the indicated gas

Experiment	Gas	k ± S.E.
1	NO	0.01390 ± 0.00017
2	H_2S	0.01276 ± 0.00023
3	NO then H_2S	0.01329 ± 0.00028
4	H_2S then NO	0.01235 ± 0.00030

ecent evidence on the activities of the
icals constitutes further reason for sus-
ting there are distinctions possible between or among the long-lived radicals. Up to the present time, we have taken the position that reduction of the free-radical class by either NO or H_2S accomplishes the same effect quantitatively. In table 1 are data indicating that this is not quite true. H_2S always reduces the value of k to a level slightly below that seen after NO treatment. If radicals only are involved, some are more available to H_2S than they are to NO. It is difficult to conceive how this can come about because of chemical reactivities, for if, as we think, reduction of radical concentration is brought about by H_2S by its donating a hydrogen atom

$$R\cdot + H : SH \rightarrow R : H + \cdot SH$$

this effect should be less efficient than

$$R\cdot + \cdot ON \rightarrow R : ON$$

that does not require an energy-requiring abstraction process before it occurs. For this reason, it is conceivable that the difference indicated by these data, if the two gases are involved only with radicals in these instances, is one of position, or location. This argument implies that H_2S or $H\cdot$ atoms can move more freely than $NO\cdot$ molecules; effectively, the radiation-induced radicals are closer to H_2S than they are to NO.

On the other hand, we note that NO either alone, or preceding H_2S treatment, has the same effect. H_2S, either alone or preceding NO treatment, has the same effect, and always a more efficient one than NO. If the "position" argument in the paragraph above were true, H_2S treatment whether before or after NO treatment should exert a greater effect.

Alternatively, the results can mean that there is a qualitative difference in the Class III species. There may be two long-lived species, both of which are removed by H_2S in a similar manner; when they react with NO, on the other hand, a difference is seen. One of them becomes harmless, and the other becomes harmful. This difference, which is the likely explanation of the results, is not the difference indicated by the two EPR spectra, nor is it the possible difference inferrable from the behavior of the unannealable radicals, but rather it must be considered evidence for a third subdivision of the Class III component.

The thermal behavior of the free-radical component

After irradiation

The postirradiation effects of heat on the lifetime of the radical component have been measured (Webb, Powers and Ehret, '60). The degree of reversal of this component for any time of annealment can be described as

$$\text{Fraction remaining} = \exp(-C_T\tau)$$

where τ is time after irradiation during which the spores are held at temperature T, and C_T is the rate constant for the temperature.[3] The fraction remaining is expressed as the degree of reversal observed relative to the maximum degree possible. The maximum obviously is the Class III component, and, as pointed out above, is measured experimentally as

$$k_{III} = k_{N_2} - k_{NO}$$

Therefore, when spores are irradiated in N_2 and exposed to O_2 thereafter, when no appreciable annealment takes place within the irradiation interval, and post-irradiation annealment takes place at temperature T for time τ, the expression relating survival to dose (equation 2) becomes

In this, the time τ is the duration of a nealment at temperature T after dose has been delivered at a low temperatur or in a short interval, so that no appre able annealment could have occurred du ing this period.

When time of annealment is zero, th constant is not reduced at all; when it very long, $\exp(-C_T\tau)$ becomes very sma and the inactivation constant is very near $k_I = k_{NO}$—i.e., the free radical compone disappears.

During irradiation

Actually, it is impossible to irradiate any but the lowest temperatures witho causing some annealment during the tim of irradiation. It is true that if the irradi tion interval is short, and if the temper ture is not high, the effect of the annea ment is small and may not be appreciabl and the observed response curve may n depart from equation (1). Most of our e periments are designed to minimize th effect, and thus to obviate these departure

In some instances, however, it is n possible to make minimal the degree annealment during the irradiation interva as, for example, in our recent neutron e periments (Powers, Ebert and Moore, u published) in which the dose rates we necessarily low, and, therefore, the irradi tion times were very long, sufficiently lon (up to 10 hours) to suspect that appre able annealment of the Class III comp nent might have taken place. In other i stances when the temperature is high annealment rates are large enough t

$$S = 1 - \Big[1 - \exp\Big(-\}(k_{NO}) + (k_{N_2} - k_{NO}) \cdot \exp(-C_T\tau)\{ \cdot D\Big)\Big]^n \qquad (3$$

cause an important reduction in the Class III component even in short irradiation intervals. Accordingly, for a description of these instances, a modification of equation (3) is required that will take into account the degree of annealment occurring while the spores are being irradiated. Furthermore, the expression is certainly less general without this modification.

Obviously in these instances the time of annealment τ in the annealment function $\exp(-C_T\tau)$ is not equal to the irradiatio time t, since the radicals formed at diffe ent times during the irradiation interva are exposed to the annealing effects of hea different periods of time. Rather, the tion of the maximum annealable rate

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[3] In this publication (Webb, Powers and Ehre '60) the relationship between C and T was show to conform to the Arrhenius relation $-\ln C$ A/RT, with A being about 16 kcal. Obviously th value substituted in equation (3) would define th expression more explicitly physically, but we r tain C in this discussion for simplicity of expre sion.

(Class III) that is effective over the irradiation interval is given by the average value from 0 to *t*,

$$\bar{f} = t^{-1}\int_0^t \exp(-C_T\tau)d\tau = \}1 - \exp(-C_T t)\{/C_T t$$

refore, the inactivation constant to be d to k_I in equation (3) to give the ap- nt constant is

$$k_{N_2} - k_{NO})(\}1 - \exp(-C_T t)\{/C_T t)$$

hich *t* is the time of irradiation, C_T is

the rate of annealment of radicals, and $(k_{N_2} - k_{NO})$, as above, is that portion of the inactivation constant that is Class III, the long-lived free radical component, or, the rate of formation of these. Equation (3) can now be stated in the more general form

$$S = 1 - \left[1 - \exp\left(-\}(k_{NO}) + (k_{N_2} - k_{NO})([1 - \exp(-C_T t)]/C_T t)\{ \cdot D\right)\right]^n \quad (4)$$

describes the survival observed after *D* given over time *t* at temperature *T*. here is, in addition, postirradiation an- ment at temperature *T′* for time *t′*, then ression (4) becomes

which takes into account annealment both during and after irradiation.

This equation (4) can be applied to previously obtained response curves showing dose-survival relationships at temperatures

$$= 1 - \left[1 - \exp\left(-\}(k_{NO}) + (k_{N_2} - k_{NO})([1 - \exp(-C_T t)]/C_T t) \cdot \exp(-C_{T'} t')\{ \cdot D\right)\right]^n \quad (5)$$

h enough to demonstrate the anneal- nt effect. In figure 5 the two curves wn are equation (4) in which k_{N_2} is 318, k_{NO} is 0.0169 and $C = 0.160$ min.$^{-1}$ temperature 343°K; and k_{N_2} is 0.0326, is 0.0174 and $C = 0.500$ min.$^{-1}$ for °K. The inactivation constants were en from figure 2; the points are experi- ntally observed. It is evident that the is very good, and equation (4) can be sidered a description of the inactiva- n process.

We can note that when one estimates ctivation constants from such responses using the nondescriptive function such equation (1), then the value of *k* will d low in same temperature ranges (these the values appearing in figure 2). ually one of the components, k_I, in- ases with temperature; the other, k_{III}, pears to be almost temperature-independ- in this temperature range as far as its luction by X rays is concerned. But after tain radiation times at these tempera- es, survival is the result of the opera- n of an inactivation constant that ap- rs to be less than the sum of these two. is is a misleading answer caused by the rmal reduction of part of one compo- nt, which, with respect to its formation, temperature-independent.

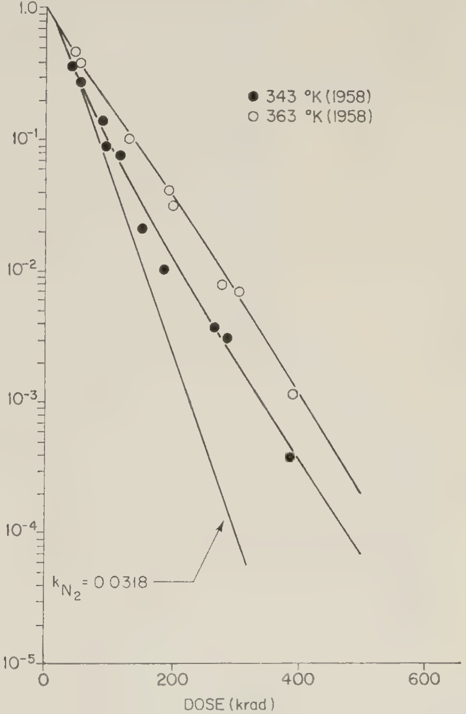

Fig. 5 The solid lines represent the curves described by equation (4) when the constants of the equation are those indicated in the text. The points are experimental ones taken from the studies upon which the paper by Webb, Powers and Ehret ('60) is based.

Estimation of reversible component from single-response curves

The division of the inactivation constant into smaller components, and the recognition of the fact that these components may behave differently under various experimental circumstances makes understandable certain complex response curves. For instance, in our experience we have often observed that at higher temperatures the survival curve is such that as dosage increases, the efficiency of the radiation decreases, i.e., survival at higher doses is higher than expected. We have previously proposed (Webb, Powers and Ehret, '60) that this is the result of thermal reversal of part of the radiation effect, and now we can demonstrate that our general expression which takes into account independent behavior of the different components d indeed constitute a valid description these survival curves in terms of th components.

It is also possible now with the use equation (4) to estimate the size of the f radical component without measuring directly, provided that an estimate of t maximum radiation sensitivity can made.

This can be accomplished by algebr manipulation of

$$S = 1 - \Big[1 - \exp(-kD) \Big]^{n}$$

to obtain

$$-k = \ln \} 1 - (1 - S)^{1/n} \{ /D,$$

Therefore,

$$(k_{N_2} - k_{NO})\Big(\} 1 - \exp(-C_T t) \{ /C_T t\Big) + k_{NO} = -\ln \} 1 - (1 - S)^{1/n} \{ / D$$

since in the general case, *k* of equation (1) is written as the left hand side of equation (6). Now there should be a constant ratio of Class III to Class I as far as induction these by X rays is concerned. Thus, letting

$$(k_I + k_{III})/k_I = Z,$$

$$-\ln \} 1 - (1 - S)^{1/n} \{ /D = k_{N_2}(1 - 1/Z)\Big(\} 1 - \exp(-C_T t) \{ /C_T t\Big) + k_{N_2}/Z$$

whence

$$-Z = \frac{k_{N_2}\Big[1 - \Big(\} 1 - \exp(-C_T t) \{ /C_T t\Big)\Big]}{\Big[\ln \} 1 - (1 - S)^{1/n} \{ /D + \Big(\} 1 - \exp(-C_T t) \{ /C_T t \cdot k_{N_2}\Big)\Big]}$$

This is to say that knowing k_{N2}, *c*, and *t*, one can estimate k_{N_2}/k_{NO} for each *D* and *S* obtained experimentally, and, therefore, an apparent value for $k_I(k_{NO})$.

As an example of the application of this, consider the response curve obtained by irradiating spores at 60°C., a temperature at which appreciable reversal of Class III should be expected by the end of the irradiation interval. From the temperature studies (Webb, Powers and Ehret, '60) an estimate of k_{N2} at 60°C. can be made, and it is used in equation 7 as 0.0313 kr^{-1}. At different values of *S* and *D*, with $C_{60^\circ} = 0.07$ $minute^{-1}$, we obtain values of *Z* as in table 2. The values obtained vary about the mean value for *Z* of 1.257, indicating in these series of experiments $k_I = 0.0249$. In figure 6 the experimental points a plotted and the fitted curve is superin posed. The fit is coincident.

While this apparent value of k_I allowe equation (4) to describe the experiment points, the difference between this value k_I (0.0249) and the one ordinarily o served (0.0155) is very large, and was u expected. Immediately, therefore, th preparation was tested directly for k_I b annealment at 80°C. after irradiation i N_2 at 25°C. and the measured value wa found to be 0.0245, in perfect agreeme with the value obtained from the singl response curve.

It is important to note that the term "a parent value of k_I" is used. The explan tion of this result is not that the real valu

TABLE 2

Values of k_{N_2}/k_{NO} calculated from expression (7) from an experiment at 60°C., k_{N_2} being 0.0313 for X rays, $c = 0.07$ min.$^{-1}$

S	D	t	k_{N_2}/k_{NO}	Mean
	kr	*min.*		
0.01	165	12.8	1.280	1.257
0.001	246	19.2	1.180	
0.0001	335	25.6	1.220	
0.00001	445	32.0	1.350	

$_I$ (the oxygen-independent part of the
ponse) has changed, but rather that the
of Class III as measured by thermal
ealment has changed. We have pointed
frequently that the annealment phe-
nenon depends upon circumstances and
tors that must be controlled, and vari-
results are sometimes seen. On the
er hand, the variability observed after
tirradiation treatment with NO has
n very small. This probably means that
h respect to its formation, Class III is
ependent of the experimental variables
ng manipulated in these experiments;
with respect to its disappearance before
ction with oxygen, while it is insensitive
hese variables in regard to scavenging
NO, it is sensitive in regard to thermal
ealment. Insofar as the variables in
se experiments are concerned, there are
reasons recognized for variations in
value of k_I.

Therefore, the apparent value that k_I
es in this experiment is a reflection of
increase at the expense of the Class III
nponent. The value of k_I at 60°C. is
176 kr^{-1} (Webb, Powers and Ehret, '60);
refore, the apparent value of 0.0249
nus this value gives 0.0073 as the mag-
ude of the decrease of Class III under
conditions of the experiment. Its ex-
cted size was 0.0313 − 0.0176 = 0.0137;
therefore, is 0.0064/0.0137, or 47% of
usual value.

Experimental verification of this kind of
ange in Class III is currently being ob-
ned. Studies in this laboratory point to
riations in trace amounts of water as the
ise of variability in size of the anneal-
e component. We show (Tallentire and
wers, '61) that direct removal of water
vents subsequent thermal annealment,
hough the NO component may not be

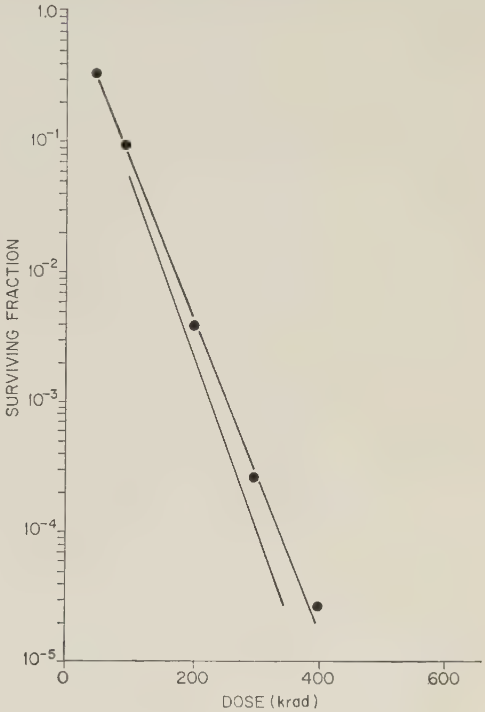

Fig. 6 A survival of dry spores irradiated at 60°C. The solid curved line represents the curve described by equation (4) after estimation of apparent k_I with expression (7). The straight line without points is the expected curve if no annealment had taken place during irradiation.

affected. It should be expected that intermediate amounts of trace water should result in intermediate degrees of thermal annealment. This method of calculating indirectly the size of the annealable component from the individual response curves will allow more rapid analysis of the factors controlling it.

With similar algebraic manipulations we can solve directly for k_I, and can evaluate it if a good estimate of k_{N_2} can be made. In this instance, the complex equation reduces to

$$-k_I = \frac{\left[\left(\ln\}1-(1-S)^{1/n}\{\right)/D+\left(\}1-\exp(-C_Tt)\{/C_Tt\right)\cdot k_{N_2}\right]}{\left[1-\left(\}1-\exp(-C_Tt)\{/C_Tt\right)\right]}$$

In very recent experiments with 2 M neutrons (E. L. Powers, M. Ebert, and Moore, unpublished data), in which the dose rate was necessarily low, and the irradiation times long, a definite departure from the expected response was observed at high doses. Using expression (8) to evaluate k_I at different points (various *S*'s and *D*'s) with *C* taken at 0.0024 minute^{-1} at 20°C. (Webb, Powers, and Ehret, '60), we obtained the values of k_I shown in table 3. The values of *S* and *D* were taken from the first portion of the response curve before deviations.

In figure 7 are shown the experimental points through which is drawn the response curve in the form of equation (4) with k_{N_2} as 0.030 and apparent k_I as 0.009.

CONCLUSIONS

The dry bacterial spore has revealed to us that there are many ways in which this cell responds to radiation; even the single biological effect that we measured in our studies is accomplished in a number of different ways. The fast, oxygen-independent portion can be subdivided into two effects, the fast oxygen-dependent portion appears at the present time to be a single entity, and the long-lived free radical class may be divisible into three different subunits, a possible total of 6 currently recognized components of radiation-induced effects that precede and lead to biological damage.

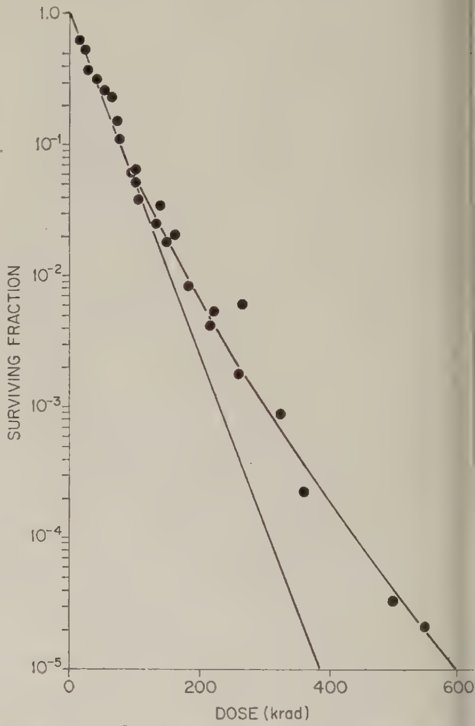

Fig. 7 The survival of dry bacterial spo after exposure to 2-Mev neutrons in N_2. T solid curve line is equation (4) after estimation $k_I + k_{III}$ from the initial portion of the survi curve, and the apparent k_I from expression 8 the text (Powers, Ebert and Moore, unpublis data). The straight line, without points, sl 0.030 kr^{-1}, is the extension of the initial par the curve.

TABLE 3

Values of k_I estimated from expression (8) with known k_{N_2}, and $c = 0.0024$ min.$^{-1}$, 2-Mev neutrons at 20°C.; 1000 rad/min.

S	D	t	k_I	Mean
	kr	*min.*		
0.0064	200	200	0.0133	
0.0029	250	250	0.0075	
0.00096	300	300	0.0092	
0.00040	350	350	0.0087	0.0090
0.00018	400	400	0.0082	
0.000079	450	450	0.0083	
0.000057	500	500	0.0062	
0.000010	550	550	0.0107	

t has been revealed also that some of
se elements may act independently and
ave differently under different circum-
nces, and thus contribute to the biolog-
l effect to different degrees at various
es. Fortunately we have been able to
late them to measure their independent
haviors, thus allowing the study of their
eractions with one another when they
at the same time. In this paper it is
own that with the knowledge of the
lependent behavior of the components
radiation damage, one can explain the
ponse of a population of cells in which
e biological effect is the result of the
tions of all. Without the division of the
sponse into components, and the sepa-
e analysis of each, this would not have
en possible, since there are forward and
ck effects, reversible and non-reversible
rtions, and the reversion rules seen so
r are not simple ones. In the instance we
ve examined in detail, that of thermal
versal, the explanation fits the observed
sult; therefore, the assumption of non-
teraction between the components is al-
wed. We should expect to see interest-
g circumstances in which interactions
nong certain of the components will be
dicated; I believe our method of ap-
oach will enable us to analyze these
teractions.

This approach is not to be confined to
e spore among the dry biological sys-
ms. There is much evidence in the liter-
ure that other systems have the prop-
ties of the bacterial spore, and we expect
at many of the same rules apply in these
stances. It is certain that in the dry seed,
ocesses similar to those described for
e spore are operating, and that it will be
ssible eventually to define critically the
diation response as we have done for the
ore. The H_2S effect, the external O_2
fect, the low-temperature effect (Nybom,
ustafsson and Ehrenberg, '52), the post-
radiation O_2 effect (Adams and Nilan, '58;
aldecott, Johnson, North and Konzak,
7), the NO effect, the existence of radi-
als (Sparrman, Ehrenberg and Ehren-
erg, '59; Conger and Randolph, '59)
nd the postirradiation heat effect (Kemp-
n and Maxwell, '41; Caldecott and Smith,
2; Konzak, Curtis, Delihas and Nilan,
0; S. Wolff and A. M. Sicard, unpublished
data), have been demonstrated in separate studies in several kinds of seeds by different authors. Although there are difficulties with this material, as for instance the apparent low permeability of the seed coats to gases, there are, on the other hand, attractive advantages, among them being biological end-points not existing in the spore, such as chromosomal (Adams and Nilan, '58; S. Wolff and A. M. Sicard, unpublished data) effects.

Nor should we expect that this general approach cannot be applied to metabolizing cells, such as mammalian cells handled in various ways. With recent improvements in quantitation, precision is being obtained and reproducible radiation constants are being observed (Hewitt and Wilson, '59; Elkind and Sutton, '60; Puck, Marcus and Cieciura, '56). They should be divisible into subunits or components.

Not until the response of the cell to radiation is partitioned will the reasons for the response observed be understood. Intuitively we know that many things must happen in an irradiated cell; so we know at the beginning that even for a relatively simple situation like survival of bacteria, a single number describing sensitivity has limited usefulness. The aim must be to delimit and isolate the separate contributing processes and controlling circumstances, in order to understand, at a later time, what they do in concert.

ACKNOWLEDGMENT

The assistance of Mr. Merlin Dipert in the computations is acknowledged with thanks.

OPEN DISCUSSION

R. B. SETLOW: Can you give us an estimate as to the chemical composition of the spores and an estimate as to which chemical species show these free radicals?

POWERS: No, sir.

HOWARD-FLANDERS[4]: Are there sulphydryl compounds in these dry spores?

POWERS: We know that there are. V. Vinter ('60) has a number of publications on the cystine content of bacterial spores (*B. cereus and megaterium*).

These compounds could very well be playing a part, say, for instance, in the value of n of our general expression describing inactivation.

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[4] P. Howard-Flanders, Yale University School of Medicine.

STEWART[5]: Were your spores lyophilized?

POWERS: No, these were dried, slowly at first, in order to obviate ice formation. Freezing apparently changes the radiation sensitivity in our system. Then we tried to dry to reproducible radiobiological effect.

STEWART: How do you dry?

POWERS: We dry them routinely by pumping on them at about 1 mm for about an hour, and then putting them over a desiccant.

STEWART: There has been some information published on the possibility of greatly varying the moisture content in the spore, an apparently dry spore, by equilibration over various desiccants, which changes its water content.

POWERS: We have one of the world's experts on this matter in our group, Dr. Tallentire. We are aware of the problems.

BILLEN[6]: I wonder what the effects of the various treatments might have on the metabolic processes involved in spore germination. If the pretreatments do affect such processes might not this cloud the interpretation of the effects on survival you have observed. After all, the pretreatments, i.e., drying, freezing, H_2S exposure, etc. may initiate reversible or irreversible damage which may only become manifest when the spores start to metabolize.

POWERS: Yes, this is a proper thing to worry about. However, may I underline the fact that the reversal processes we are talking about concern physico-chemical reversions of some sort. They have nothing to do with reversion of some biochemical damage that might be seen at the time of spore germination.

BILLEN: Yes, but these treatments will very definitely influence posttreatment growth and metabolism.

POWERS: What the treatments do will influence things that prevent the biochemistry proper to colony formation. These treatments are 12 and 14 hours before germination and, therefore, before biochemical activity.

BILLEN: Yes, but hydrogen sulfide probably penetrates into the spore and perhaps brings about changes in some of the cell constituents. It is an interpret tion that ignores this type of variable th worries me.

POWERS: H_2S changes it by influen ing the Class III radicals. This is definiti

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ological After-Effect and Long-Lived Free Radicals Irradiated Seeds[1]

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he size of the experimental materi- in the two preceding papers and in own follows in an orderly sequence n small to large. Dr. Kirby-Smith has n primarily working on effects in mole- s and pure compounds; Dr. Powers s with larger objects, the ~1 μ in diam- bacterial spores. And I have been king for almost 4 years on a biological ct that is actually large enough to see, ely, barley seeds.

ow, in spite of the fact that each of re working on very different objects, in very different ways, I believe that phenomena we are studying are closely ted and very similar, if not identical. own belief is that we are working on same thing. And, as evidence of this, fer the fact that we find a great deal rgue about with one another when we together and talking about our work.

ou may be interested to know what pens when these simple physical and sical-chemical phenomena are looked not in nice clean molecules or in an in- esimal mote of life, but in a big, nasty, fusing biological object like a seed, all the built-in biological crankiness uch an object. I believe that biologists e an instinctive desire, nay, an in- ent demand, to have concrete demon- tion that some known physical or mical phenomena actually operate in ear-cut manner in a macroscopic bio- cal system. The disillusionment one ers in trying to extrapolate from simple sico-chemical theory to the biological ality is familar to most of us. Yet, for subject considered in this morning's sion, I believe the extrapolation is in t cases quite straightforward, accepta- and to a biologist, gratifying. As evi- ce, I offer some of the information we have garnered by our experiments on biological damage and long-lived free radicals in irradiated seeds. Dr. Calvin Konzak of Washington State University is the man who has very kindly supplied me (and a lot of other people) with the barley seeds I use, and I am happy to be able now to publicly thank him.

Surprisingly enough, many of the same things do happen in the seeds. And it was this, initially, that trapped me into working on them. I had the hope that it might be possible to do radiobiological experiments for which moderately simple physical or physico-chemical explanations would suffice, and in fair measure, this has proved to be possible.

Curtis *et al.* ('58) first showed that damage to dry barley seeds increased with increasing storage time after irradiation. In a typical experiment, dry seeds are irradiated and then either planted immediately (soaked in water immediately), or stored for some time post-irradiation before planting (soaking in water). The change in amount of biological damage for a given dose by storage after irradiation is called the "after-effect" (After-effect = $\text{Damage}_{\text{stored}}/\text{Damage}_{\text{immediate}}$). "Immediate" damage may be increased by storage as much as 20-fold, but more commonly by about 3 to 5 times. A post-irradiation decrease in damage is what Powers calls "restoration," and is equivalent to his type III effect.

Biological damage in irradiated barley seeds can be measured as damage to seedling growth (height a seedling attains in ~7 days of growth), survival of the seed to a mature plant, chromosomal aberrations,

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[1] This work was supported in part by a United States Atomic Energy Commission grant, AT-(40-1)-2579, to the author.

or mutation. The various damages are related. Most of the experiments measure damage to seedling growth, though we have also measured chromosome aberrations. Seedling height is inversely related to radiation dose and gives a typical sigmoidal curve against dose down to about 10–20% of the unirradiated (control) height, where it strikes a dose-independent plateau. The dose-dependent part of the seedling growth is due to cell division plus enlargement; the dose-independent part to cell enlargement only. Thus, survival of seed embryo cells which divide after irradiation is what is being measured. To compare the modification by different treatments, sensitivity is estimated from dose curves as the dose required to reduce the height to 50% of the control, or, from the seedling heights at a single dose.

I have cherished the notion, and still do, that the biological after-effect we see in the irradiated dry seeds is due to decay of the long-lived free radicals, derived from radicals initially generated by the radiation, and persisting after the irradiation is over. Much of Kirby-Smith's molecular results, and Powers' spore results, can be interpreted the same way. Using conventional ESR methods (initially on Dr. Kirby-Smith's machine), we were able to detect free radicals in seeds and seed embryos post-irradiation. The radical signal resides mostly in the protein and nucleic acid fraction of the seeds (none in fats and oils), is a broad signal showing no fine structure, has a half-width of 27 gauss and g value of 2.0040, i.e., it looks like a general featureless protein radical signal (Conger and Randolph, '58). More recently we have found fine structure in the radical signal and changes in fine structure following changes in conditions (gas, temperature, etc.).

My efforts have been devoted to testing the effect of various treatments (water, oxygen, gases, temperature, etc.) on these post-irradiation free radicals and on the after-effect phenomenon, to see if the one can be explained by the other. In general, the two agree quite well.

My working hypothesis is that treatments which (1) increase the yield of free radicals present immediately after irradi-
ation, and (2) increase the decay of th
radicals, also increase the biological aft
effect. In brief, radical decay post-irrad
tion is what causes the after-effect.

A simplified summary of the fact
which alter the biological after-effect m
be useful here. After-effect is enhanc
or is maximal when the seeds: (1) a
dry, (2) have oxygen present, (3) are
about room temperature, (4) are irra
ated with low ion-density radiation (X o
rays), and (5) increases with increasi
storage time. After-effect is diminished
prevented by: (1) presence of water, (
absence of oxygen, (3) presence of ra
cal scavengers (e.g., nitric oxide) and
donors, (4) storage at low temperatu
(below ~ −70°C.), and (5) high ion de
sity irradiation. There may not have be
time to grasp these generalities from t
more detailed presentations by Kirby-Sm
and Powers, but for all three materia
theirs and mine, they are in the ma
valid.

The same factors, mentioned abo
have been tested for their effect on ra
cal production and decay. Treatme
which either prevent production of ra
cals which persist to be seen postirrad
tion, or prevent their decay postirrad
tion, also prevent after-effect.

I am grateful to the Department of Bi
ogy of Brookhaven National Laboratory
their hospitality, and generous assista
in allowing me to do this ESR radical wo
at their laboratory.

Dryness. I feel it is important to e
phasize that what I am and will be d
cussing here is effects in *dry* see
This dryness is also true of the work p
sented in the two previous papers. T
questions which were directed to D
Kirby-Smith and Powers indicate that t
point regarding dryness apparently is
properly appreciated, or perhaps was
sufficiently emphasized. The very dry c
dition we are able to obtain in our ma
rials is what permits the biological cha
ber of horrors Powers and I are able
create, and get away with, in our expe
ments—very high vacuum and high pr
sures, temperatures from −270° to o
+100°C., treatment with the toxic
NO, the violently poisonous H_2S, and e

pure bromine, and so on. What I k we are doing (and the way the er should think of this) is some sort adiation chemistry, or physical chem-, or even solid-state physics, but with ogical materials. And the dry seeds ld no more be considered biologi-during the radiation and these dread-treatments, than is Tutankahmen's mmy. So soon as we wet the materials, treatments themselves become lethal. n figure 1, I have summarized some of biological and free-radical results in ls. The left axis (solid line curves) is biological damage or relative sensitiv-and comes, as I mentioned previously, n comparisons of the 50% height es, taken from dose curves. Damage, ensitivity, of seeds sown (plunged into water) immediately after irradiation is the base line (relative damage = 1). The first graph (A) shows that damage of dry seeds in air increases (about 3-to 5-fold) with increasing storage time at +25°C. After-effect develops relatively slowly (about 90% saturated after 10–15 hours), and continues to develop at a very low rate out to 4–5 weeks. This leisurely pace of development is, of course, far slower than Powers finds for his bacterial spores (full development in ~10–15 minutes or less) and is in keeping with our more southerly latitudes, compared with that of Chicago.

The relative number of radicals (dashed lines) seen postirradiation (as per cent of those seen immediately) is indicated on the right axis.

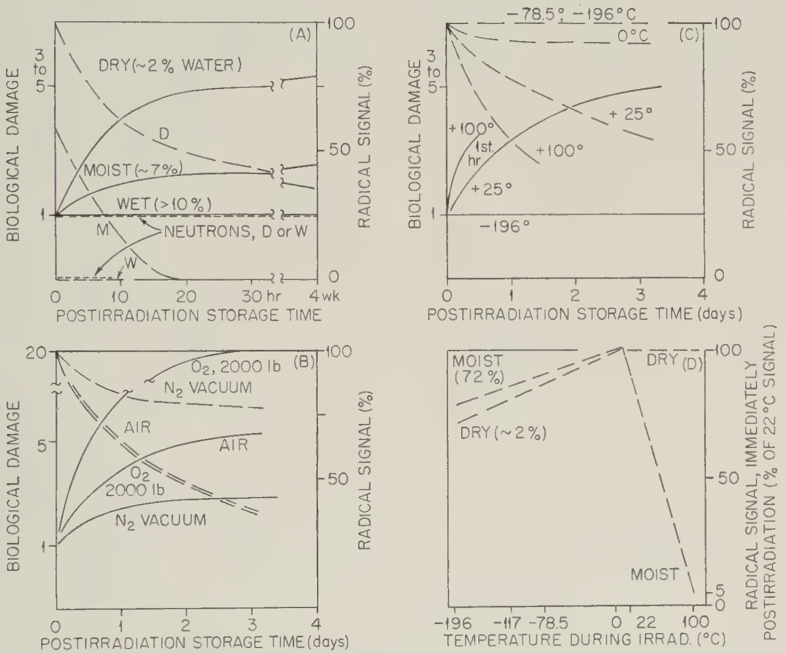


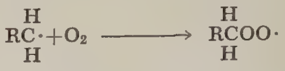
Fig. 1 Influence of various environmental conditions on development of biological after-effect (or, on relative biological sensitivity), and on production and decay of free radicals in irradiated barley seeds. Solid lines, left axis: relative biological damage (relative sensitivity). Dashed lines, right axis: free radicals (relative numbers). (A) Effect, vs storage time, of water content of seeds, and of ion density. Seeds irradiated and stored in air, at indicated water contents, with Co^{60} gamma rays, and with uranium fission fast neutrons (short dashed lines). (B) Effect, vs storage time, of oxygen content. Seeds gamma-irradiated and stored dry (2% water). (C) Effect, vs storage time, of postirradiation storage temperature. Seeds gamma-irradiated dry, in N_2, at 25°C.; stored dry, in N_2. All were stored for entire time at indicated temperature, except for the 100° biological damage results, which were heated to 100° for the first hour postirradiation, the rest of the time at 25°. (D) Effect of temperature during irradiation on yield of radicals seen immediately postirradiation (at 25°C.). Seeds gamma-irradiated dry, in air.

The graphs illustrate the generalizations given previously. For example (graph A), after-effect damage in dry seeds increases at the same rate and to about the same degree as the radicals disappear. Wet seeds, on the contrary, which develop no after-effect, have no postirradiation radicals.

It was first shown by Curtis that with high ion-density radiation (fast neutrons), in contrast to X or γ rays, there is no after-effect in seeds irradiated either wet or dry. We were able to show that following neutron irradiation there were no detectable radicals, even after very large doses, (up to 120 times as large as the dose of X or γ rays which clearly give detectable radical signals). I believe this fact is critical. But I interpret this identical effect (no after-effect and no radicals) of wetness and of high ion density as being of totally different origin. When the seeds are wet, no radicals can persist because molecules are free to move about and recombine very rapidly. With neutrons on the other hand, the initial ionization events—and hence the radicals produced from them—are so close together that most can recombine even if the material is dry. Thus, in the seeds, to have postirradiation radicals and after-effect, we must prevent radicals from disappearing by recombination during irradiation; and we can do so, firstly by generating them remotely from one another, by low ion-density radiation, and then by keeping them apart long enough for them to persist after the radiation, as by dryness. I shall return to this subject.

Immediately below, figure 1B, is illustrated the effect of oxygen on damage and on radicals. Anoxia reduces decay and damage below that found when stored in air. Here again disappearance of radicals and development of damage are correlated. The exception is for high pressures of oxygen; this enhances radical decay but little, if at all, yet it enhances damage by a very large amount. I suspect, and have some evidence for it from chromosomal analysis, that the high pressure of oxygen has a biological synergistic effect, something we had found previously with dry pollen (Conger and Fairchild, '52a and b).

As to oxygen, from these results a
from others, we believe that postirrad
tion free radicals may, under some con
tions, be disposed of harmlessly. But
they be exposed to oxygen, they are c
verted to organic peroxyl radicals



$$\overset{H}{\underset{H}{RC}}\cdot + O_2 \longrightarrow \overset{H}{\underset{H}{RC}}OO\cdot$$

and either the formation of the pero
radical, or of something derived from
is the event which leads to additional
logical damage. Powers has shown
same thing for his bacterial spores.
should note, however, that he finds
after-effect in the anoxic state, whereas
do, even though less than when oxygen
present (fig. 1B).

The two graphs on the right (figs. 1
and D) illustrate the effect of temperat
during or after irradiation. Postirradiat
heating enhances damage and decay (
1C). The biological results are firm
the cold and +25°C. curves, but are
yet for the biological experiment at +10
I cannot yet say if the 100° heat shock
1 hour enhances damage or only increas
the rate of its development. In any ca
the biological damage increases as d
radical decay, as shown for the other tre
ments. These results disagree with Powe
"heat annealment" results in his
spores, in which heat-shock postirradiati
reduced the damage.

The experiment (fig. 1D) on effect
temperature during irradiation on yield
radicals seen immediately after was und
taken because of our general notion th
any agent—water, high temperature, e
—which increased molecular mobility (i
decreased the rigidity of the solid glas
or crystalline seed embryo system) a
increased radical recombination; the
result would be a concomitant reducti
in radicals surviving to the end of irra
ation, and of damage which could sub
quently develop. The results appear
bear this out. At above zero temperatu
radical disappearance is very greatly
hanced in the moist seeds, as expect
but not in the dry, even up to +100°. Ra
cal survival or preservation in dry se
will doubtless decline at still higher te
peratures, but we have small hope of be

to do biological experiments in this ge. The temperature-dependence of ical survival at below zero temperatures light, and less for moist than for dry. feel that the mechanism by which ical yield is modified by temperature at and at high temperatures is different. s is discussed later, in connection with er content, which shows the same nomenon.

Figure 2 shows the effect of one particu- factor—water content—upon the num-

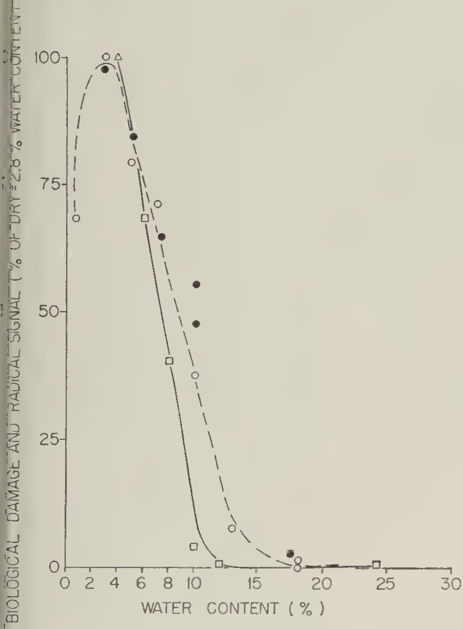


Fig. 2 The influence of water content of nma-irradiated barley seeds on: ●, ○, - - -, mber of free radicals seen immediately post-adiation, and, □, ——, amount of postirradia-n biological damage (after-effect) which will elop (when stored long enough, 7 days in to allow full development). Seeds gamma-adiated, in air, at 25°C.

r of radicals present immediately after adiation (dashed line), and on the hount of after-effect damage which will velop in seeds irradiated and stored (4 ys, long enough to develop full after-ect) at the indicated water contents. rtis *et al.* ('58) first showed that radio-nsitivity of seeds was independent of ater content if they were sown immedi-ely postirradiation; but if stored, for a ne long enough for the after-effect to saturate itself, sensitivity varied with water content as shown on the curve—decreasing from a maximum at about 4% water to none at 12% or greater. Now when we looked at the radical yield immediately postirradiation (the dashed line) we found a striking similarity. After-effect damage is just about proportional and at the same water contents, to the number of free radicals available for postirradiation decay.

But we found an additional and totally unexpected result, namely, when we went to *"super dry"* states, i.e., to water contents below that (~3–4%) studied by Curtis, the curve for radical yield went down to approximately 60% of the maximal yield found at a water content of ~3–4%.

Since this turnover of the radical curve in the "super dry" region below 4% water (down to 0.9% water) seemed so critical, we sought for it in biological damage experiments and have been able to demonstrate it. From experiment to experiment, our biological curves shift back and forth somewhat on the per cent water axis, but they do bend over, as I hoped we could find.

I was very puzzled initially for any mechanism to explain this curve, first for the radical yield and then confirmed for damage, which goes through a maximum at some point (~3% water in my material), and declines both above and below. As a working hypothesis, I have assumed that what counts in these reactions is the degree of molecular mobility in the seeds. If, as I suspect, the dry seed embryos should be thought of as glassy or crystalline solids, then we should speak of the rigidity of the solid as being the critical factor. Since, of course, I feel I am dealing with radical reactions, I really mean radical mobility, not of the initial radiation-induced radicals, but the mobility of the long-lived organic free radicals derived (by many steps) from them. Many of our results, not only on water content, but also on temperature during and after irradiation, are interpretable on this basis. Of course, this simple model of molecular mobility may not be correct (and in some particular cases I feel that other explanations are required) but in most cases it is useful.

Curves such as figure 2—a maximum at some point, and declining values above and below—have been found in radiochemical reactions. The most pertinent to my case, I believe, are results on the effect of various factors on radiation-induced degradation or radical formation in organic monomers and polymers. In these materials, curves of the same shape were generated by changes of such factors as: molecular size, degree of cross-linkage, concentration of solute, temperature during and after irradiation, and some others. The one characteristic all these factors influence in common is molecular (radical) mobility, i.e., the rigidity of the solid. Some change it by altering the molecular size, others by altering the rigidity of the medium the molecules (radicals) find themselves in.

Consider the maximum at a certain irradiation temperature (e.g., fig. ID). At temperatures above the maximum, radical yield (and after-effect) diminish because movement is so free that most radicals have a range of movement which permits encounter leading to recombination and disappearance (believed harmless). At temperatures below the maximum, the medium is so rigid that molecular recoil of the sister fragments from an ionization event is prevented; the radical pairs are held so close together that most disappear by instantaneous recombination. It can be seen that this last argument reduces to almost the same one used for the high ion density results from fast neutrons; the difference is that instead of being held together as with cold, with neutrons, the radicals are generated in large numbers so close together along the proton track that lateral (and instantaneous) recombination of adjacent radicals becomes a highly likely event. And similarly for the other factors—there is some optimal "rigidity" which is low enough so that the radical pairs are permitted to move apart and become remote at generation (reducing recombination), but high enough
they are prevented subsequently from fr
movement and encounter with one a
other. In our own experiments, we inte
pret our water content results, and o
temperature during and temperature aft
results, on this basis.

So, in conclusion, we have a model
which several processes are going on. Rad
cals once produced may recombine, or
act with other biological molecules. If th
recombine, the process is mostly harmles
If they react with other molecules, a fra
tion will react with biologically critic
molecules and so cause more damag
Factors which generate radicals close t
gether (high ion density irradiation)
hold them together (very low temper
tures, dryness, very rigid states) enhan
rapid recombination and result in fe
radicals postirradiation and in little afte
effect. Factors which permit freedom
movement (high temperature, high wat
content, low rigidity) have the same resu
On the other hand, given conditions duri
irradiation which permit survival of ra
cals, then conditions afterwards whic
permit free movement enhance recomb
nation and reduce damage; those whic
reduce movement reduce recombinati
and permit more reaction resulting in mo
damage. And an important part of the r
action process leading to more damage
the conversion of the long-lived radica
to peroxyl radicals by reaction with oxyge

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e Effects of Ultraviolet Light on the Biological d Physical Chemical Properties of xyribonucleic Acids[1]

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E. GAJEWSKA, D. LANE [5] AND P. DOTY
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e effect of ultraviolet light (UV) on
pounds of biological interest has been
ubject of numerous investigations. In
cent excellent and comprehensive re-
, Shugar ('60) has described and evalu-
the effects of UV on nucleic acids and
onstituents. At the *chemical level* it
d appear that of the deoxyribonucleic
(DNA) bases, the pyrimidines are al-
l more readily than the purines (Wier-
wski and Shugar, '61). Recent work
kers and Berends, '60, and Wang, '60,
pertaining to the photochemistry of
eic acids has opened a new dimension
e field by invoking thymine-dimer for-
on when this pyrimidine is irradiated
UV either in the frozen state or in its
natural habitat, DNA (in solution)
cker, Dellweg and Weinblum, '60;
kers, Ijlstra and Berends, '60; and
ker, Dellweg and Lodemann, '61). At
physical chemical level of UV-induced
damage, many changes have been
cted and described, but the most sen-
e thus far would appear to be the low-
g of the thermal denaturation tempera-
of native DNA (Marmur and Doty,
. At the *biological level,* the inactiva-
of transforming factor DNA, while not
ensitive as the killing of bacteriophage,
rs the most convenient means of study-
at the macromolecular level, the end
lts of UV irradiation on DNA. Another
ul biological index of UV damage to
is the progressive increase in com-
nent-fixing ability of the irradiated
as a function of dose (K. Herrington,
ublished results; Marmur and Gross-
n, '61).

he purpose of the present communica-
is to present data bearing on the ef-
s of UV on DNA at the macromolecular level, obtained by the use of biological, chemical and physical-chemical tools, with the hope that their combined approach may be useful in explaining the effects that occur *in vivo.*

MATERIALS AND METHODS

Transformation

Transformation of *Diplococcus pneumoniae* (R-36A) was carried out according to the method of Fox and Hotchkiss ('57). *Bacillus subtilis* (168) transformation was carried out as described by Spizizen ('59).

Deoxyribonucleic acid

DNA was isolated from exponentially grown cells by the method of Marmur ('61). Hybrid N^{14}–N^{15} *Escherichia coli* B DNA was isolated from cells first grown for many generations in $N^{15}H_4Cl$ as the sole nitrogen source followed by one generation (doubling of the turbidity) in N^{14} medium (Meselson and Stahl, '58). Other DNA samples used in this study were isolated from T_2r^+, *Streptomyces viridochromogenes* (94), *Shigella dysenteriae* (15), *D. pneumoniae, Pseudomonas aeruginosa* (B-23), *Clostridium perfringens* (87b), *B. subtilis, E. coli* strains I and K-12, and *Serratia marcescens.* The values listed by

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Belozersky and Spirin, '60, were used for the base compositions of the DNA from these organisms.

CsCl density-gradient centrifugation

The method employed is similar to that described by Meselson, Stahl and Vinograd ('57). DNA in approximately 5.7 M CsCl was centrifuged at 44,770 RPM for approximately 24 hours and the banded DNA photographed using ultraviolet absorption optics. By using a standard DNA of known density (isolated from *Cl. perfringens* or N^{15}-labeled *Ps. aeruginosa*), the buoyant densities of the banded DNA were calculated after first tracing the photographs with a Joyce-Leobl microdensitometer.

Ultraviolet irradiation

DNA solutions were irradiated using an Hanovia Sc-2537 mercury resonance lamp (unless otherwise stated) fitted with an acetic acid (43%) filter (Oster and McLaren, '50). The solutions were exposed to UV in a quartz vessel of 1 cm path length while the solution was mixed by a small quartz stirrer driven at approximately 300 RPM. The light intensity was varied by altering the distance from the lamp housing and was calculated by means of an uranyl oxalate actimometer (Daniels, Mathews, Williams, Bender and Alberty, '56).

Viscosity

The intrinsic viscosity of the DNA samples was determined using a four-bulb multigradient viscometer of the Ubbelodhe type to allow an empirical extrapolation to zero gradient. The solutions were dissolved in 0.15 M NaCl plus 0.015 M Na citrate and the values are expressed in deciliter per gram (dl/gm).

Sedimentation

Sedimentation coefficients of native and denatured DNA samples were determined in a Spinco Model E ultracentrifuge fitted with ultraviolet absorption optics. Solutions were centrifuged at a concentration of 20 μg/ml in centrifuge cells fitted with Kel-F centerpieces. Molecular weights of denatured DNA samples were determined from their sedimentation coefficients using the relationship established by Eigner
('60); for native DNA samples, the r
tionship established by Doty, McGill
Rice ('58), was used:

$$s_{20,w} = 0.063M^{0.37}$$

Formamide denaturation

To denature DNA prior to CsCl dens
gradient centrifugation, samples were
lyzed against two changes (one hour ea
shaking at 37°C.) of 100 volumes of
98% formamide (Fischer Reagent Ch
ical) containing 0.15 M NaCl plus 0.01
Na citrate at neutral pH (Marmur and T
'61). The formamide was removed
dialysis prior to centrifugation.

Photoreactivation

Photoreactivating enzyme from bak
yeast was generously supplied by Drs.
Rupert and R. M. Herriott. Photoreacti
tion was carried out at 37°C. in a therr
statted water bath at a distance of 2
from a Sylvania 15-watt "cool-wh
(F15T8-CW) lamp. The reaction was
minated by immersing the sample in
ice bath.

Sonic treatment

Sonic degradation of DNA was carr
out in the presence of the free-radical tr
ping agent, AET, as described by I
Marmur, Ephrussi-Taylor and Doty, '58.

Formaldehyde reaction

In order to test the reactivity of nat
and UV-treated DNA with formaldeh
(Fraenkel-Conrat, '54), 2.5 ml of the D
solution in 0.15 M NaCl plus 0.015 M
citrate held at 50°C. in glass-stoppered
vettes in the Beckman DU spectrophot
eter chamber was mixed with 0.2 ml o
37% formaldehyde solution. By follow
the absorbance (OD) at 270 mμ at z
time (T_0), at any time (T) during
course of the reaction and after com
tion of the reaction (F), the reaction-r
constant (k) could be derived from
slope obtained by plotting the time of
action in hours against

$$2.3 \log (OD_F - OD_T)/(OD_F - OD_{T_0}).$$

Acid titration

To DNA at a concentration of 20 μg/
in 0.05 M NaCl, at 25°C., was added
creasing amounts of HCl. After each a

of acid, the pH and absorbance were rmined. The absorbance relative to obtained at the neutral pH was then ted as a function of the pH. The pH alf denaturation is referred to as $pH_{1/2}$ nville and Geiduschek, '60).

EXPERIMENTS

Effects of ultraviolet irradiation on the biological activity of DNA

. Comparative sensitivities of trans- ning DNA and phage viability. Since bacteriophage genome presents a larger ss-section (Hershey and Burgi, '60), many more vulnerable areas to the e UV dose than does a genetic marker ated on a transforming DNA molecule, tt *et al.*, '58), it would be expected that of phage viability would be more sensitive than the inactivation of transforming activity. This has been found to be the case (fig. 1). The capacity for expression of the genetic units of the transforming DNA is initially inactivated by UV irradiation with single-hit kinetics at an approximate rate of one hit to the streptomycin or indole locus per 6–10 phage lethal damages. This is approximately of the same order of magnitude for the inactivation of the A cistron of r^+_{II} and of T_4 phage viability (Krieg, '59).

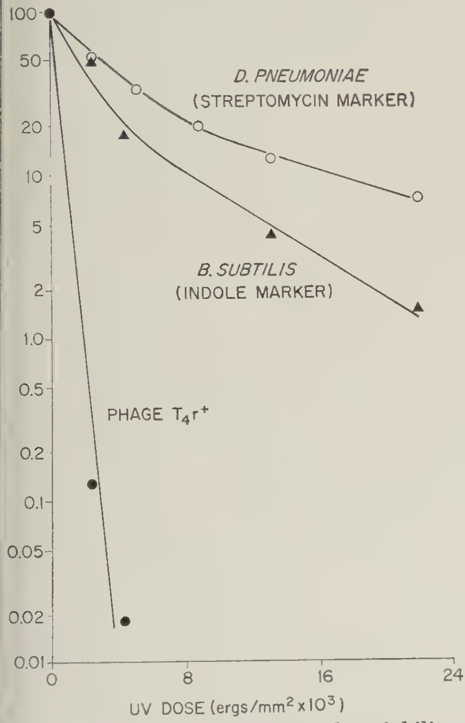


Fig. 1 Effect of UV light on the viability of + and the transforming activities of *D. pneu- niae* and *B. subtilis* DNA. The bacteriophage 2×10^9) was suspended in 0.01 M phosphate ffer, pH 6.9 containing 0.001 M Mg^{++} for the adiation. Transforming DNA was irradiated at concentration of 40 µg/ml. Irradiation of the A in the phosphate-magnesium buffer or in 5 M NaCl plus 0.015 M Na citrate gave es- tially similar results.

2. *Differential sensitivities of genetic markers of transforming DNA isolated from the same source.* The differential inactivation of genetic markers of transforming DNA has been reported previously (Zamenhof, Leidy, Greer and Hahn, '57; Lerman and Tolmach, '59; Rupert and Goodgal, '60; and Stuy, '61). In general, no correlation has been found between the UV sensitivity of genetic markers associated with transforming DNA and their susceptibility to inactivation by thermal denaturation. Since the latter property is related to the average base composition of the DNA molecule (Marmur and Doty, '59; Doty, Marmur and Sueoka, '59), while the former is related to the differential sensitivities of the individual markers, no direct correlation would be expected, since the DNA molecules of bacterial origin are rather homogeneous in base composition (Sueoka, '59). This does not rule out the possibility that the differential UV sensitivities are related to specific sequences of the bases for different markers. Figure 2 shows the UV sensitivity of the unlinked markers controlling histidine and indole utilization of *B. subtilis* DNA.

It was thought of interest to study the inactivation of linked genetic markers that reside on the same transforming DNA molecule. D. M. Green and A. Ravin (unpublished results), have shown that the ery_2 and ery_3 markers of *D. pneumoniae* which control the resistance to the antibiotic erythromycin, behave in transformation experiments as if they were linked. Using their strains of this organism, it was found that the DNA carrying the linked ery_2 and ery_3 markers as well as the unlinked streptomycin marker displayed a higher UV resistance of the ery_2 than of the ery_3 marker. Both of these markers

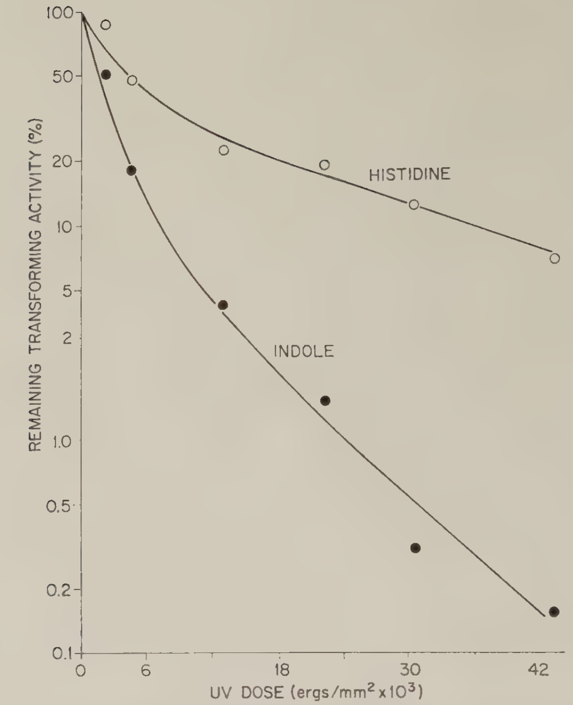


Fig. 2 Effect of UV light on the transforming activity of *B. subtilis* DNA. DNA isolated from wild type cells of *B. subtilis* (168) was irradiated at a concentration of 17 μg/ml and then assayed using strain SB1 (obtained from Dr. E. Nester) requiring indole and histidine (unlinked) for growth.

were more resistant than the linked ery$_2$-ery$_3$. Streptomycin marker sensitivity approximately equaled ery$_3$ marker sensitivity (fig. 3).

It would be difficult to attribute the large differences in UV sensitivities of the ery$_2$ and ery$_3$ markers to variations in average base composition since the markers reside on the same DNA molecule (and thus would not be expected to display proportionate differences to UV sensitivity in average base compositions in the marker regions). Also, the variations in the UV sensitivity of the linked markers cannot be due to differences in the size of the DNA molecule with which the genetic markers are associated since they are both on the same molecular unit. These differential UV inactivations may be due to: variations in the size of the genetic marker or of the surrounding regions which may be necessary for effective genetic exchange; different sequences of the bases in the DNA molecule which may favor certain photochemical lesions within selected regions akin the favoring of the "hotspots" of mutation sites of bacteriophage (Benzer, '61); t position of the genetic marker along t DNA molecule which may be an importa contributing factor if energy transfer p cedes the inactivation event, etc. At t moment it is difficult to evaluate the co tribution of either or all of these pos bilities. However, the first possibility, th of marker size, is subject to evaluatio Lacks and Hotchkiss, '60 and Rogers a Hotchkiss, '61 have proposed that the re tive size of the genetic units of transfor ing DNA can be estimated from the thermal stability *below* the denaturati temperature as well as from their tra forming efficiency. Both these criter point to the ery$_2$ marker occupying smaller region (and thus a smaller U sensitive target) than the ery$_3$ marker.

3. *Effect of molecular weight of DNA UV sensitivity.* A proper evaluation of t UV inactivation of transforming activ

res some knowledge of the molecular
of the DNA that has been irradiated.
ading conclusions may be arrived at
nterpreting differential inactivation
if the irradiated samples may vary in
(Ellison and Beiser, '60). Also, the
ibution of the size of the DNA mole-
to the rate of inactivation may shed
light on the factors influencing the
e of the UV dosage-response curve.
this relationship in mind, a study was
rtaken to evaluate the molecular
ht effect for the inactivation of several
nked markers of *D. pneumoniae*. DNA
his study was subjected to sonic vibra-
which has been shown to cause double
n scission of the molecules (Doty, Mc-
and Rice, '58). Sonic vibration alone
cause a loss in transforming activity
to a lowering of the molecular weight
size too small to satisfy the requirements for cell entry and/or the disruption of the marker region (Litt *et al.*, '58). A homologous series of sonically prepared DNA samples varying in molecular weight were made from the same DNA (initial molecular weight: 8.5×10^6). A dosage-response curve was obtained for each sample. The results for the marker conferring streptomycin resistance are shown in figure 4. Similar results have been obtained with the genetic marker conferring resistance to bryamycin and erythromycin. It is immediately apparent that the same genetic marker, residing on smaller molecules, is more resistant to UV. The quantum yield (McLaren and Takahashi, '57) for the UV inactivation of the streptomycin marker using a preparation of M.W. 3.2×10^6 was 3.9×10^{-5} mole/Einstein for the fast component of the dosage-response curve and 9.8×10^{-6} mole/Einstein for the slow component.

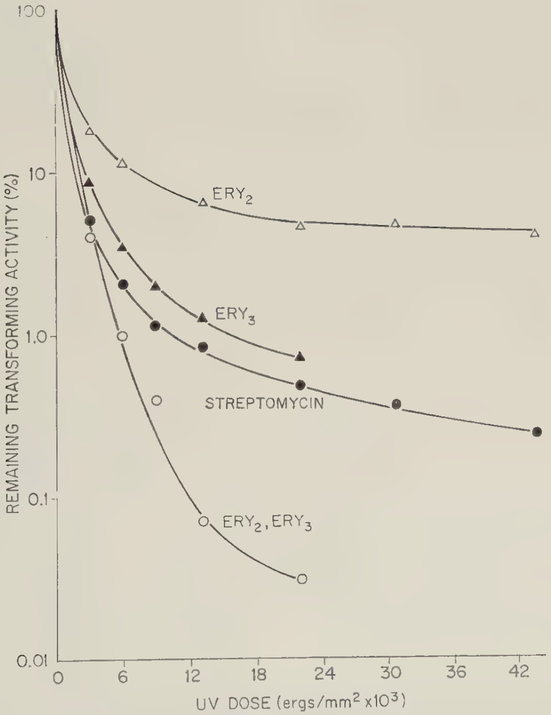


Fig. 3 Effect of UV light on the transforming activity of unlinked and linked genetic markers of *D. pneumoniae* DNA. DNA isolated from a strain of *D. pneumoniae* R_x ery_2ery_3str (obtained from Drs. A. Ravin and D. M. Green) resistant to a high level of erythromycin ($ery_2 + ery_3$) as well as streptomycin was irradiated at a concentration of 40 μg/ml in 0.15 M NaCl plus 0.015 M Na citrate. The irradiated samples were assayed using R-36A as the receptor strain. The same strain of *D. pneumoniae* was used as the receptor strain and mutants thereof for the source of transforming DNA.

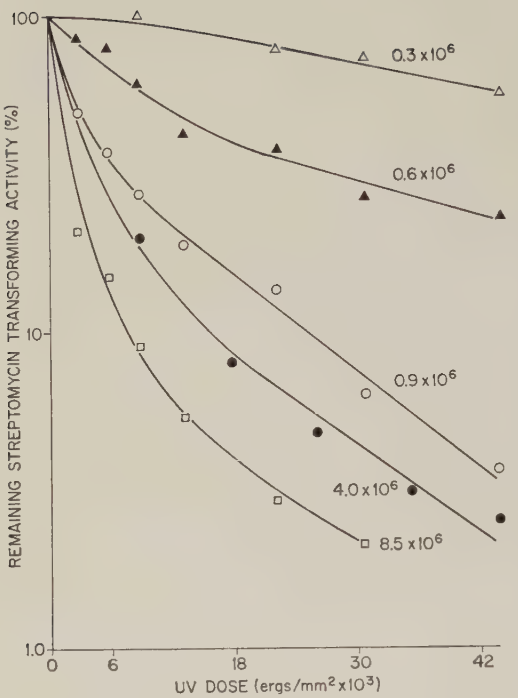


Fig. 4 Effect of UV light on the transforming activity of sonically treated *D. pneumoniae* DNA of varying molecular weights. DNA was sonically treated at a concentration of 220 μg/ml in 0.15 M NaCl plus 0.015 M Na citrate containing 2.5% AET. The AET was then removed by dialysis and the samples irradiated at concentrations of 36–38 μg/ml in 0.15 M NaCl plus 0.015 M Na citrate.

By invoking the differences in the size of the DNA molecules irradiated rather than the heterogeneity of the genetic units controlling similar functions inferred from the differential inactivation curves of the marker in different column elution fractions, the results of Ellison and Beiser, '60 are readily explicable.

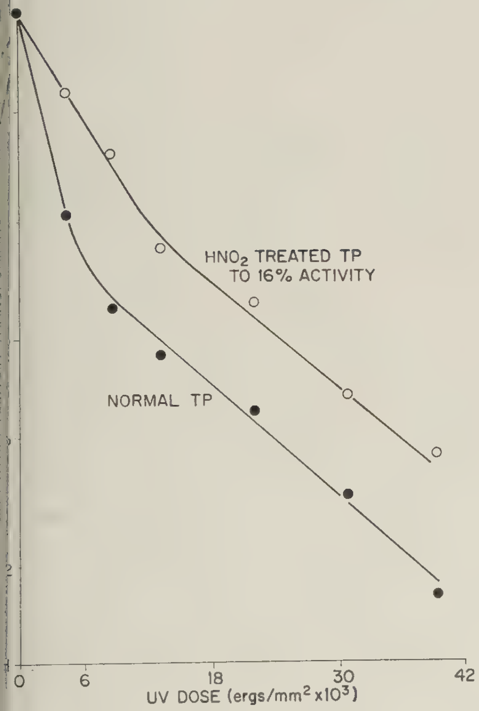
The results shown in figure 4 may help to explain, in part, the shape of the inactivation curves of transforming DNA. It is possible for instance that, when isolated, DNA molecules carrying a specific genetic marker are located on molecules heterogeneous as to molecular weight. The genetic marker associated with larger molecules would be inactivated at a faster rate than those associated with the smaller molecules and thus give rise to a dosage response curve with a continuously changing slope. However, the fact that DNA isolated from irradiated cells also results in a somewhat similar, multicomponent cu (Stuy, '61), diminishes the contribution this explanation. However, if the D isolation procedure is carried out in a m ner that would lead to a wide distribut of molecular weights, one might obtai different inactivation curve than would obtained with a more carefully isola and thus more homogeneous preparati

The effect of molecular weight on UV sensitivity fits two different hypothe that have been proposed to explain irradiation inactivation curves. One is t of L. S. Lerman (personal communi tion) who believes that the absorption a photon can result in a photochemical sion at a site removed from the absorpt site or lost at certain singularities of DNA molecule. He invokes the concept excitation transfer and assumes that lifetime of the excitation is related to distance between single sites such as p tochemical lesions. Thus, by increas the UV dose (which is mimicked by sonic treatment), the interval betwe singularities is decreased, resulting in shorter lifetime of the excitation and he a higher resistance to irradiation.

The other explanation, extrapola from the findings of the inactivation bacteriophage, is based on the hypothe that genetic exchange is facilitated by tegrity of the DNA molecule extending yond the marker regions. UV induced sions in these regions which can be rescu by recombination with the genome of recipient organism, as well as the mar region itself, would reduce the chances effective genetic exchange. The probabil of rescue by recombination from lin damages becomes progressively smaller the UV lesions occur closer to the mar region (Krieg, '59). At high doses, the li ing slope would thus represent the in tivation due to a direct hit, the probabil of rescue having been greatly diminish Either or both explanations may serve explain the shape of the UV inactivat curve for the loss of biological activity transforming DNA or of other biolog material.

4. *Effect of HNO_2 pretreatment of D on UV sensitivity.* Nitrous acid treatm of transforming DNA results in a loss biological activity most likely because

leamination of the amino groups h participate in the normal hydrogen ng of specific complementary base (Zamenhof, Alexander and Leidy, Bautz-Freese and Freese, '61). Prement of *D. pneumoniae* DNA with so that 16% of the streptomycin forming activity remained, rendered emaining activity more resistant to nactivation than an aliquot which had een pretreated (fig. 5). No significant ges in molecular weight resulted from INO_2 treatment since the sedimentacoefficient remained unaltered. It is ble that the chemically induced "le-" resulting from the HNO_2 treatment mimic those produced by either UV ment itself (Guiduschek, '61; Marmur Grossman, '61) or sonic degradation e DNA all of which lead to the same esult in rendering the biological inactivation by UV more resistant than native, untreated DNA.



g. 5 Effect of UV light on the transforming ity of normal and nitrous acid treated *D. moniae* DNA. Normal DNA and DNA which been exposed to HNO_2 (Zamenhof, Alexander Leidy, '53) to 16% remaining streptomycin forming activity, each at 15 μg/ml in 0.15 M plus 0.015 M Na citrate were irradiated and ed for their ability to transform to strepto-
n resistance.

5. *Effect of irradiation of host cells on the assay of UV-treated DNA.* It would seem possible that the UV-induced lesions of DNA, if they occur within the regions of the molecule that are homologous to the corresponding molecules in the host cell, might be repaired at some stage in the act of transformation. By UV irradiating the recipient cells, lesions might be induced (Rupert, '61) which, if they corresponded to those in the irradiated transforming DNA, would prevent their repair. This was found to be the case (fig. 6). The assay of UV irradiated transforming DNA from *D. pneumoniae* shows a higher recovery on non-irradiated than on irradiated cells. It would of course be naive to suggest that the above explanation is the only reason for the observed results.

6. *Effect of UV on the inactivation of homologous and heterologous DNA.* By thermally denaturing a mixture of streptomycin marked DNA with an excess of wild type DNA and subsequently annealing the material at a temperature below the melting temperature (T_m), it is possible to convert the homologous genetically marked DNA to the heterologous renatured state (J. Marmur, D. Lane and P. Doty, unpublished results; Marmur and Lane, '60). The question that can be asked is whether this heterologous DNA (one strand carrying the marker conferring streptomycin resistance and the other the wild type allele) is more or less sensitive to UV than is homologous renatured DNA. In making the comparison, it is necessary to provide the proper control; the homologous DNA alone was denatured and annealed at the same total concentration that was employed in preparing the heterologous DNA. Figure 7 shows that both the heterologous DNA and the renatured homologous DNA have the same sensitivity to UV. This conclusion is in keeping with the evidence that in transformation, only one of the two strands is believed to be effective in carrying out a phenotypically observable transformation of the recipient cell (J. Marmur, D. Lane and P. Doty, unpublished results). The results, at variance with those of Herriott, '61, may be explicable either by the differences inherent in the biological

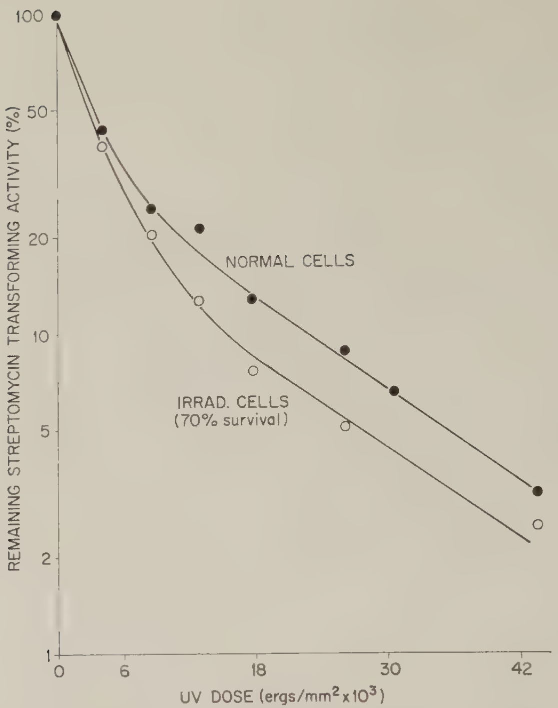


Fig. 6 Effect of UV light on the transforming activity of *D. pneumoniae* DNA assayed on normal and UV treated cells. DNA isolated from streptomycin resistant cells of *D. pneumoniae* was irradiated at a concentration of 40 μg/ml in 0.15 M NaCl plus 0.015 M Na citrate and then assayed on normal streptomycin sensitive cells and on UV treated cells, which on the average, retained 70% of their viability. The results on the irradiated cells represents the average of several experiments.

material used to carry out the experiments or by the fact that the UV inactivation of the heterologous DNA was compared by Herriott to that of native homologous DNA instead of renatured DNA. It is known (Doty, Marmur, Eigner and Schildkraut, '60) that the thermal exposure used in the conditions for denaturation and renaturation causes some backbone breakage of the DNA. Since there is a molecular weight effect on UV sensitivity (see section A-3), it is necessary that the UV inactivation curves be carried out on samples that had been exposed to the same thermal conditions.

B. *Effects of ultraviolet irradiation on the physical chemical properties of DNA*

1. *Effect of UV on the stability of the DNA helical structure.* Since UV is known to cause photochemical alterations of the pyrimidine residues of DNA with relatively high quantum yields (Shugar, '60), and since the integrity of the base residues necessary to maintain the stability of t double-stranded DNA molecule, it was l lieved possible that UV irradiation wou weaken the stability of the structure. Tl effect, most likely produced as a result localized interference with normal hydr gen bonding, was followed by a number techniques.

(a) *Lowering of the melting tempe ture T_m, of DNA.* The helix-coil transiti of DNA can be readily followed by the i crease in relative absorbance which a companies the disruption of the hydrog bonds and the resultant separation of t strands. The midpoint of the absorban rise, the T_m, is indicative of the stability the DNA molecule. When DNA is expos to varying doses of UV, the T_m shifts lower temperatures and the relative abso ance change (hyperchromic shift) is minished (fig. 8). The lowering of the reflects a weakening of the helical D

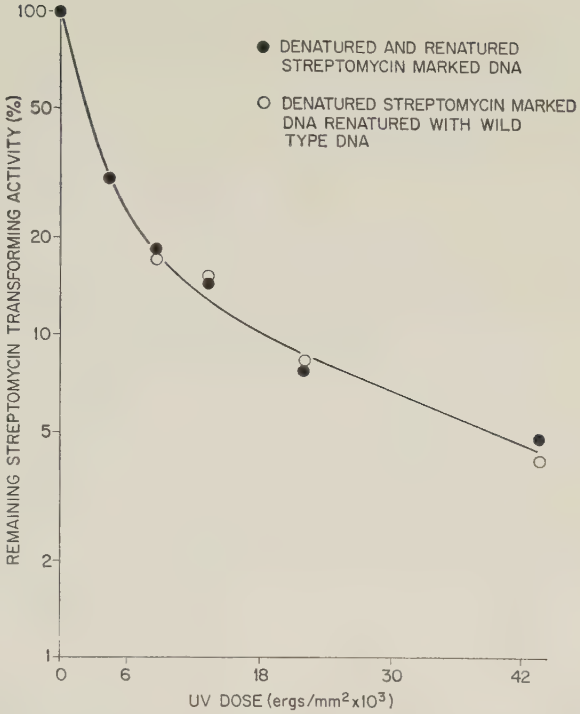


Fig. 7 Effect of UV light on the transforming activity of renatured homologous and heterologous DNA. Heterologous renatured DNA was prepared by denaturing and annealing streptomycin marked together with wild type DNA (1:3), each at a concentration of 40 μg/ml in 0.3 M NaCl plus 0.03 M Na citrate. The homologous renatured DNA was prepared by renaturing each DNA (wild type and marked) *separately*, at the same concentration and in the same solvent, and mixed before irradiation. The irradiation was carried out in the same solvent and aliquots removed and assayed after varying UV doses for their ability to transform to streptomycin resistance.

cture, while the inability to attain the
al increase of absorbance may reflect
e initial denaturation caused by the UV
alone and/or the inability to separate
pletely all the DNA molecules that
e suffered UV damage. The latter ex-
ation would seem plausible if cross-
ing of the two complementary DNA
nds could take place (see section B-3b).
he lowering of the denaturation tem-
ture of irradiated DNA might be help-
n explaining the results of Zamenhof,
y, Hahn and Alexander, '56. They
d that genetic markers associated with
ophilus influenzae DNA were "un-
ilized" when the DNA was exposed to
since it was found that the transform-
activity was more thermal-labile than
rradiated DNA. Their observed "un-
ilization" of the genetic material most
y reflects the weakening of the double-
al structure by UV as shown above.

The results of figure 8 can be summarized for irradiated *D. pneumoniae* DNA as well as DNA isolated from *Ps. aeruginosa, Serratia marcescens* and *E. coli* by plotting the T_m against the UV dose (fig. 9). Slight variations are observed in the rate of fall of the T_m depending on the base composition of the microbial source being irradiated. *D. pneumoniae* DNA, with the highest mole per cent adenine-plus-thymine composition examined, exhibits the highest sensitivity in this test.

(b) *Lowering of the melting temperature of poly* ($A + U$). Since the synthetic polyribonucleotide copolymers mimic the properties of DNA and RNA (Doty, Boedtker, Fresco, Haselkorn and Litt, '59), it was of interest to see if the lowering of the T_m could be observed with a polymer such as poly ($A + U$). Figure 10 shows that this synthetically prepared polymer behaves in a similar manner to DNA when subjected to UV irradiation. K. L. Wierzchowski and D. Shugar (personal communication) have obtained similar results.

(c) *Raising of the acid transition range, $pH_{1/2}$.* The helix to coil transition of DNA can also be induced, at room temperature, by lowering the pH. A sharp increment in the relative absorbance occurs when native DNA molecule collapses; the p is the pH at the midpoint of the absorba increase (Bunville and Geiduschek, ' Figure 11a shows the titration curve

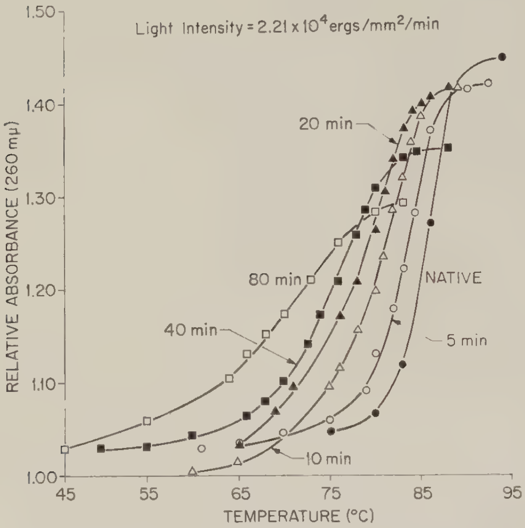


Fig. 8 Effect of UV light on the thermal denaturation of *D. pneumoniae* DNA. DNA in 0.15 M NaCl plus 0.015 M Na citrate at a concentration of 20 μg/ml was irradiated for varying periods of time. Each sample (in the same solvent) was then placed in a quartz, glass stoppered cuvette and heated in the Beckman spectrophotometer, model DU. The absorbance relative to that at 25°C. was calculated and plotted as a function of temperature of the DNA solution.

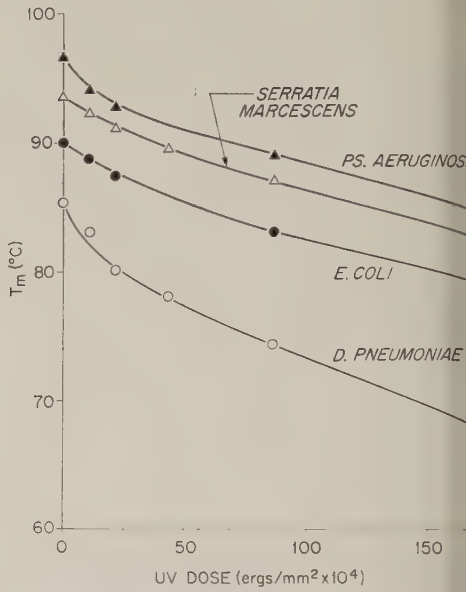


Fig. 9 Effect of UV light on the thermal turation of DNA samples varying in base position. DNA extracted from *Ps. aerugi* (67% G + C), *Serratia marcescens* (58% G + *E. coli* (50% G + C) and *D. pneumoniae* (3 G + C) was irradiated for varying doses an absorbance-temperature curve determined a scribed in the legend of figure 8. The half turation temperature, T_m, was then plotted function of the dose.

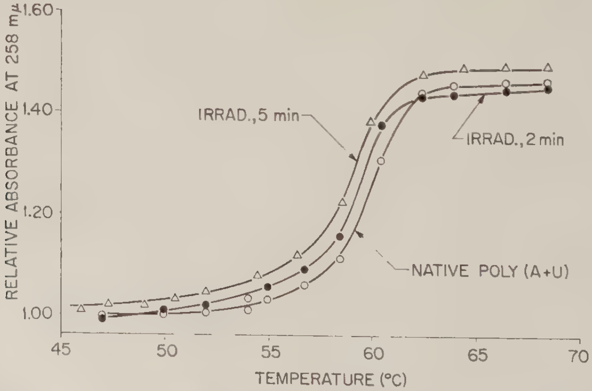
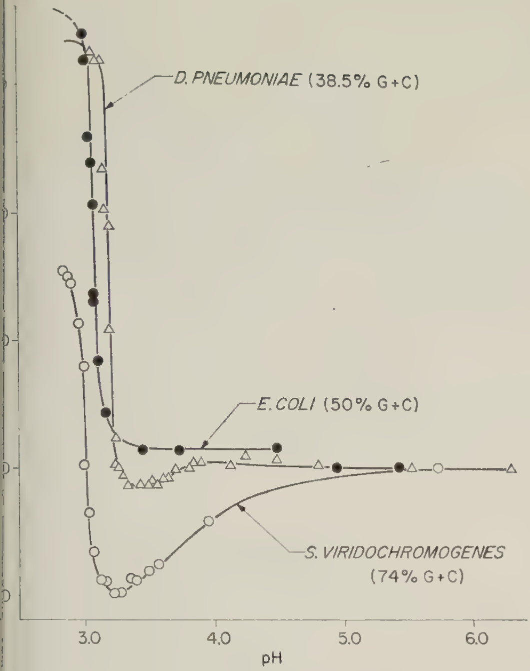


Fig. 10 Effect of UV light on the thermal denaturation of poly (A + U). Poly (A + U in 0.15 M NaCl plus 0.015 M Na citrate at a concentration of 20 μg/ml was irradiated fo varying periods of time at a light intensity of 4.4 × 10³ ergs/mm²/min. and the absorbance temperature curve determined as described in the legend for figure 8.



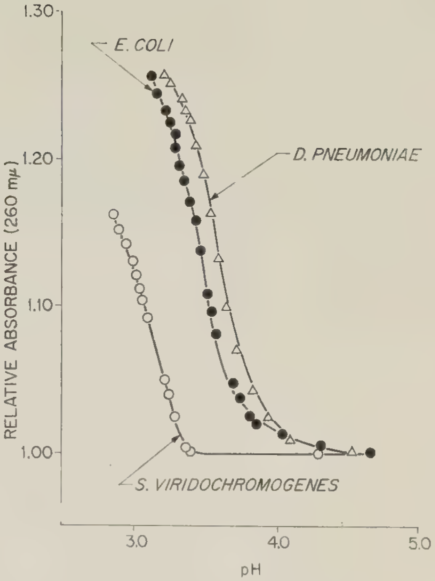


Fig. 11a Acid denaturation of various DNA samples in 0.05 M NaCl at 25°C. DNA ex- racted from the organisms listed in the figure was titrated with HCl spectrophotometrically s described in the section on Materials and Methods.

Fig. 11b Effect of UV light on the acid denaturation of various DNA samples in 0.05 M NaCl at 25°C. Each sample of DNA at a concentration of 20 μg/ml was exposed to a dose of 33×10^4 ergs/mm^2 and then titrated with HCl spectrophotometrically as described in the ection on Materials and Methods.

radiated DNA from various microbial ces which vary in base composition. dip in the relative absorbance preced- he denaturation is accentuated for the samples possessing a higher guanine- cytosine composition and is believed due to the protonation of the cytosine ues. When the same samples are ir- ated, the pH$_{1/2}$ shifts to higher values the dip due to the cytosine disappears 11b). Also the breadth of the transition ider for irradiated than for nonirradi- samples. Besides confirming the hy- esis of a weakening of the DNA struc- by UV, these results provide evidence the photochemical alteration of the sine residues.

1) *Increased reactivity with formalde-* . Denatured DNA reacts more readily formaldehyde than native DNA ossman, Levine and Allison, '61; Hasel- and Doty, '61). When *D. pneumoniae* was exposed to UV, its reactivity with aldehyde was greatly increased (fig. 12), again supporting the contention that the DNA molecules are weakened in structure and partly denatured as a result of irradiation.

2. *Effect of UV on the molecular weight of DNA.* In attempting to account for the loss in biological activity of irradiated transforming DNA, the question of a UV-induced backbone breakage was investigated by measuring the drop in molecular weight as a function of dose. The study was carried out with native and denatured DNA, 5-bromouracil (5 BU) containing DNA as well as with sonically treated DNA and with synthetic polyribonucleotides.

(a) *Lowering of the sedimentation coefficient.* When native DNA is subjected to UV irradiation, very little change is noted in the sedimentation coefficient unless the irradiated DNA is denatured. This is because any backbone breakage of one of the strands is masked unless another break occurs on the opposite strand, both breaks

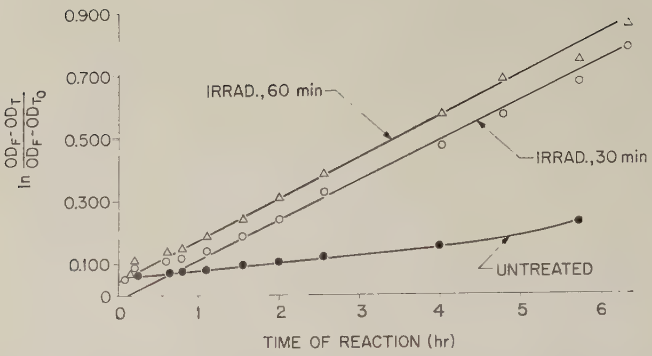


Fig. 12 Effect of UV light on the reaction of *D. pneumoniae* DNA with formaldehyde at 50°C. *D. pneumoniae* DNA in 0.15 M NaCl plus 0.015 M Na citrate at a concentration of 20 μg/ml was irradiated for 0, 30 and 60 minutes at a light intensity of 4.4 × 10^3 ergs/mm^2/min. The samples were then exposed to formaldehyde as described in the section on Materials and Methods.

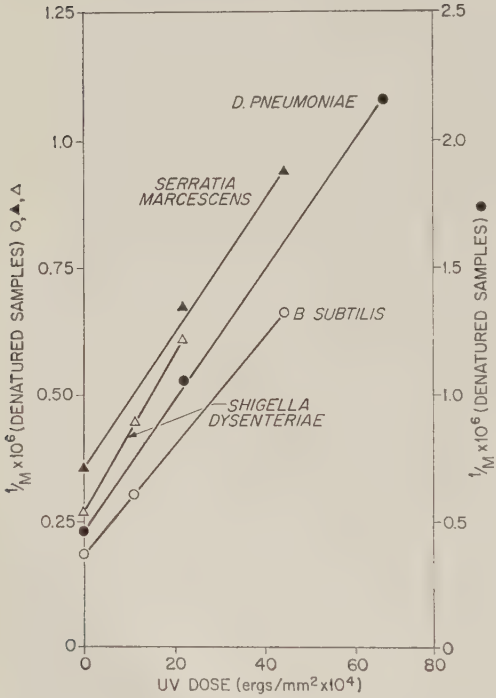


Fig. 13 Effect of UV light on the molecular weight of DNA denatured *after* irradiation. DNA from *D. pneumoniae, Serratia marcescens, Shigella dysenteriae* (53.4% G + C) and *B. subtilis* (42.4% G + C) in 0.15 M NaCl plus 0.015 M Na citrate, each at a concentration of 20 μg/ml, was irradiated for varying periods of time. Each sample was then denatured by exposure to 100°C. for 10 minutes followed by fast cooling and the sedimentation coefficient and molecular weight determined as described in the section on Materials and Methods.

occurring within several nucleotides of
another. In order to detect UV indu
single chain breaks, DNA from vari
bacterial sources was irradiated and t
denatured by exposure to 100°C. for
minutes followed by rapid chilling. The
sults are shown in figure 13. The mo
ular weights of the irradiated, denatu
samples were estimated from their s
mentation coefficients (Eigner, '6
There is no dramatic effect of DNA b
composition on UV sensitivity; howe
DNA isolated from *D. pneumoniae* wh
possesses the highest content of aden
plus-thymine (61%) of the samples
amined is the most sensitive. The quant
yield for the drop in the molecular wei
however, cannot account for the observ
decrease in biological activity (figs. 1,
5) and other explanations must be sou
to explain the irradiation results obtai
in transformation studies.

(b) *Effect of UV on denatured an*
BU DNA. If DNA is first denatured
then irradiated, there is a very minor
in the sedimentation coefficient. Howe
when the operations were carried out in
reverse order, a considerable drop in
sedimentation coefficient occurred. Th
sults are summarized in table 1. *E. co*
a thymine-requiring strain (obtained f
Dr. S. Zamenhof) was used as the so
of DNA in these studies. The incorp
tion of approximately 10% 5 BU into
DNA of this strain rendered the denatu
DNA (as well as native DNA in other

TABLE 1

Effect of ultraviolet light on denatured DNA

DNA irradiated	$s_{20,w}$	
	0 Dose	13.2 × 10^5 ergs/mm^2
E. coli I (denatured)	27.9	25.4
E. coli I (5 BU, denatured)	29.74	12.15
E. coli I (native)	27.9	15.4[1]

[1] Irradiated at a dose of 6.6 × 10^5 ergs/mm^2 in the native state, sedimented in de-atured state. Samples (native or denatured) were irradiated in 0.15 M NaCl plus 0.015 M a citrate at a concentration of 20 μg/ml. The sedimentation coefficients were determined n denatured samples.

TABLE 2

Effect of ultraviolet light on the sedimentation coefficient of synthetic polynucleotides

Compound irradiated	$s_{20,w}$	
	0 Dose	13.2 × 10^5 ergs/mm^2
Poly (A+U)	6.68	6.48
Poly U	3.85	4.38
Poly A (single strand)	4.59	4.08
Poly A (double strand)		3.91[1]
Poly C	2.14	2.25[2]

[1] Irradiated as double strands, sedimented as single strands.
[2] Dose: 3.3 × 10^5 ergs/mm^2.
The synthetic polynucleotides were irradiated at a concentration of 20 μg/ml.

nents) very susceptible to the UV-in-d decrease in sedimentation coeffi-. If this effect is of any importance plaining the increased biological sen-ty of 5 BU containing DNA to UV ra-Kubinska, Lorkiewicz and Szybal-61; Greer, '60), it would add evidence e hypothesis that the modes of inacti-n are quite different if the halogen-pyrimidine is present or absent.

) *Effect of UV on the sedimentation cient of synthetic polynucleotides.* e 2 shows that there is very little ob-able alteration of the sedimentation icient of irradiated polynucleotides at relatively high UV doses. It was nally hoped that these model com-ds could be useful in estimating the tum yield for backbone breakage. ever, their small weight (fig. 14) ren-d these polymers relatively insensitive e effects of UV with respect to de-se in molecular weight. It would be ting to speculate that the slight in-se in the sedimentation coefficient of iated poly U is due to dimer formation tion B-3b). The polymers, however, been useful in detecting other UV ced alterations such as photochemical lesions, weakening and denaturation of the double stranded structure, etc.

(d) *Effect of UV on the intrinsic viscosity.* Any backbone damage induced by UV would result in an altered configuration of the DNA molecule and would be reflected by a change in the intrinsic viscosity. Figure 14 shows the results obtained by irradiating DNA isolated from various sources. DNA from *D. pneumoniae* shows a higher sensitivity than the other bacterial samples. DNA isolated from bacteriophage T_2r^+, which had an intrinsic viscosity of 150 dl/gm and a higher adenine-plus thymine content (66%) than the other samples, exhibited the highest sensitivity of all. When *D. pneumoniae* DNA was sonically degraded, it exhibited a high resistance to changes in intrinsic viscosity by UV irradiation. This has also been noted with respect to its biological sensitivity (fig. 4).

It has been reported that the drop in molecular weight of DNA shows a marked oxygen effect during UV irradiation (Moroson and Alexander, '61). *D. pneumoniae* DNA was irradiated while nitrogen or oxygen was being bubbled through the solution and both the drop in viscosity and loss

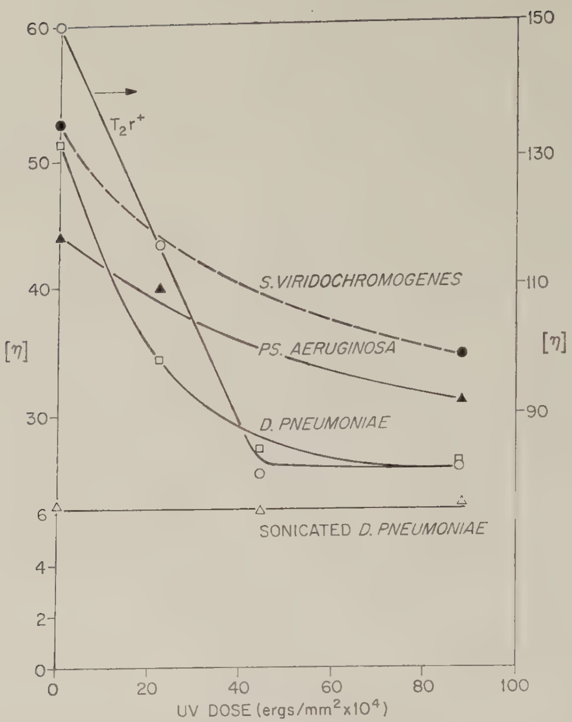


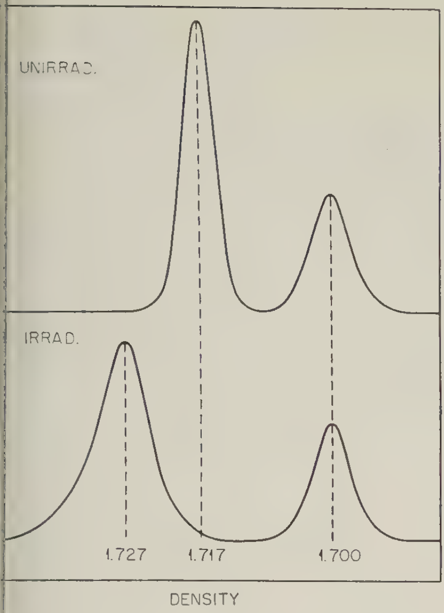
Fig. 14 Effect of UV light on the intrinsic viscosity of native and sonicated DNA. DNA isolated from the organisms listed in the figure was exposed to various doses of UV in 0.15 M NaCl plus 0.015 M Na citrate each at a concentration of 20 μg/ml. The intrinsic viscosity (dl/gm) was determined for each irradiated sample and then plotted as a function of the dose.

of biological activity followed as a function of dose. Both assays showed identical results when the DNA solution was saturated with either gas. The discrepancy in the results remains unexplained.

3. *Effect of UV on the buoyant density of DNA in a CsCl gradient.* (a) *Increase in DNA buoyant density by UV.* A readily observable effect on purified DNA caused by UV irradiation is its increased buoyant density in a CsCl gradient (fig. 15). The increase is observed for the whole population of DNA molecules and does not result in their strand separation. If either of these events took place, more than one band would be observed on the irradiation of the $N^{14}N^{15}$ hybrid *E. coli* B DNA. Figure 15 shows that only one band, with a buoyant density 0.010 gm/cm^3 higher than that of native hybrid DNA was obtained after exposure to UV irradiation. Higher doses resulted in further density increases and again strand separation did not ensue. However, DNA extracted from irradia cells, even at high doses, does not disp an increased buoyant density (E. Seam unpublished results).

When a series of DNA samples diffe in base composition were examined, it found that for the same UV dose, higher the adenine plus thymine cont the greater the density increment. Si denatured DNA also increases in buoy density on UV irradiation, the increa observed when native DNA samples v irradiated can be only partially due some denaturation. The remainder of density increase is probably related to photochemical alterations of the pyr dine residues. In the irradiation of wl cells, there would appear to be some tective action that eliminates the ph chemical lesions producing the density crease of DNA.

(b) *Cross-linking of the DNA stra* The discovery that thymine dimers coul



g. 15 Effect of UV light on the buoyant den-
of hybrid N^{14}–N^{15} *E. coli* B DNA. *E. coli* B
in 0.15 M NaCl plus 0.015 M Na citrate at a
entration of 20 μg/ml was exposed to a dose
33 × 10^4 ergs/mm^2. Both the unirradiated
irradiated samples were then centrifuged in
Cl gradient (together with a *D. pneumoniae*
standard, density 1.700 gm/cm^3) and the
ant density of the banded DNA determined.

ted from UV irradiated DNA (Wacker
l., '61) and the cross-linking of dried
s of DNA by irradiation (Setlow and
le, '54) prompted a search for a cross-
ing between the complementary strands
he DNA molecule. When DNA is ex-
d to UV doses within the range of inac-
tion of transforming DNA, a portion of
molecules becomes resistant to strand
ration when exposed to conditions that
ld normally cause denaturation (Mar-
and Grossman, '61). The results on
h UV-induced, cross-linked, formamide-
stant DNA molecules are shown in fig-
16 and 17. The cross-linked material
epresented by the tracing of the mate-
with the lower buoyant density. Figure
shows that phage DNA and, in general,
e DNA molecules that possess a high
nine-plus-thymine content, gives rise
high proportion of cross-linked mate-
when subjected to UV. *Ps. aeruginosa*
A, with a high G-C content, yields less
s-linked DNA than does T_2r^+ DNA. By increasing the dose of UV (fig. 17), larger proportions of irradiated DNA become non-susceptible to formamide denaturation. Cross-linking *between* complementary strands cannot alone account for the loss in biological activity, and it is of course possible and likely that other lesions and/or dimer formation within strands takes place (Marmur and Grossman, '61). When whole cells are irradiated and the DNA extracted, it also exhibits cross-linking between strands which can also be detected. However, at the present time it is not known what the correlation is between the percentage of the DNA forming cross-links and the cell viability. If only a few cross-linked DNA molecules result in the death of the cell, they would be difficult to detect except at relatively high doses.

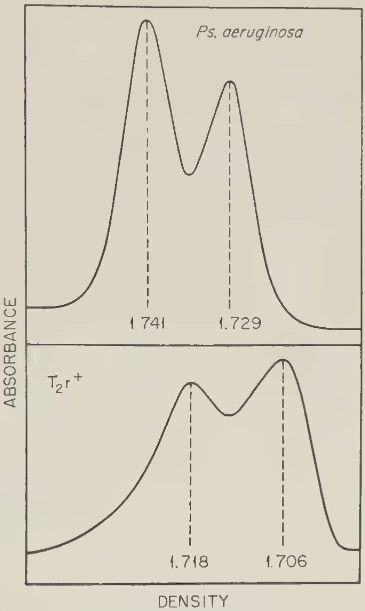


Fig. 16 Effect of UV light on the formation of formamide-resistant cross-links in DNA. DNA isolated from *Ps. aeruginosa* and T_2r^+ in 0.15 M NaCl plus 0.015 M Na citrate, each at a concentration of 20 μg/ml was exposed to a dose of 10.6 × 10^4 ergs/mm^2 at 5°C. using a G15T8 Westinghouse low-pressure mercury-discharge sterilamp. Each sample was then denatured in formamide, dialyzed and banded in a CsCl gradient. The buoyant densities were then calculated; for each of the irradiated samples shown in the figure, the lower density band represents the cross-linked material.

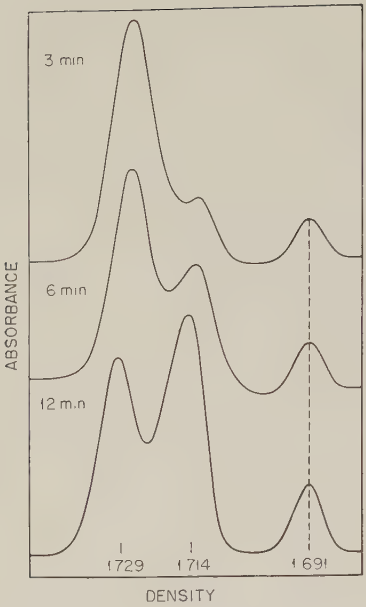


Fig. 17 Effect of UV light on the formation of formamide-resistant cross-links in *E. coli* K-12 DNA as a function of dose. DNA from *E. coli* K-12 in 0.15 M NaCl plus 0.015 M Na citrate at a concentration of 20 μg/ml was exposed at 5°C. to UV irradiation at a light intensity of 17.6×10^3 ergs/mm²/min. (same lamp as figure 16). Each sample was then denatured in formamide, dialyzed and banded in a CsCl gradient together with *Cl. perfringens* DNA which served as a standard (density = 1.691 gm/cm³).

If cross-linking results from the dimerization of specific base residues, it would most likely be preceded by certain events, such as photochemical alterations of the pyrimidine residues, localized denaturation in certain regions of the DNA molecule, etc. Evidence has already been presented in previous sections showing that the irradiated DNA molecules are weakened in structure and partially denatured, most likely resulting from the photolysis of base residues (Shugar, '60). The denatured regions might provide the conditions necessary for the proper geometric orientation of the bases prior to dimerization.

The cross-linking of the complementary strands of the DNA molecule has been demonstrated by an independent method (Marmur and Grossman, '61). By using the *E. coli* phosphodiesterase which attacks only denaturated or single stranded DNA (Lehman, '60), it was found that UV-irradiated DNA that was subsequently denatured, became progressively more refr tory to attack by the enzyme with incre ing dose. The results could be read accounted for by invoking the UV induc formation of cross-links which would p vent the separation of the DNA stran during the denaturation step and thus main nonsusceptible to the action of enzyme.

C. Reaction of UV irradiated transforming DNA

Two attempts were made to reactiv a specific genetic marker of UV irradiat *D. pneumoniae* DNA that had suffered b logical inactivation. The first attempt w to heat and anneal (Marmur and La '60; Marmur and Doty, '61) the irradiat DNA together with DNA carrying the ternate allele and the other attempt was use the photoreactivating enzyme pres in bakers' yeast (Rupert, '60).

1. *Attempt at "marker rescue" of inactivated, transforming DNA.* It w thought possible that the UV induced sions residing in any one of the comp mentary strands (other than cross-linki between strands) that result in a loss biological activity might conceivably "rescued" if the irradiated DNA was de tured and annealed in the presence of excess of non-irradiated wild-type DN The results of such an experiment shown in table 3. DNA, isolated from

TABLE 3

Attempt at "marker rescue" of ultraviolet irradiated D. pneumoniae DNA

Treatment	Relativ streptomy transform activity
1. None	100%
2. UV irradiation	7.8
3. Denature and renature no. 1	47.8
4. Denature and renature no. 2	3.5
5. Denature no. 2 and renature with wild-type DNA	3.5

D. pneumoniae DNA in 0.3 M NaCl plus 0. M Na citrate at a concentration of 40 μg/ml w irradiated and assayed before and after UV posure (samples 1 and 2). These samples w then thermally denatured and annealed (Marm and Doty, '61), and assayed as samples 3 and Sample 5 was prepared by heating and anneal with three parts wild-type DNA. Wild-type D which had been renatured by itself was added sample 4 before assay to act as the proper cont for sample 5.

moniae resistant to streptomycin was
ed to UV until 7.8% transforming
ty remained. When the irradiated
non-irradiated streptomycin marked
were denatured and annealed by
selves under optimal conditions (Mar-
and Doty, '61), approximately 50%
e biological activity remained in each
Now, when the irradiated sample
denatured and annealed with an ex-
of wild type *D. pneumoniae* DNA in
to assure the formation of hybrid
cules, there was no increase in the
gical activity over that obtained when
rradiated DNA was heated and an-
d by itself (at the same concentra-
. The assays were performed on the
of equivalent concentration of
ed DNA.

e results lead to two conclusions.
in the process of denaturation and
aling of irradiated DNA *per se* there is no increased restoration of biological activity. This indicates that any photochemical damage that is reversible by heat (Shugar, '60), does not play too great a role in the final step involved in biological inactivation of transforming DNA. Second, if biological inactivation can be related to some photochemical lesions on one of the strands, then the fact that the replacement of the complementary strand by one not treated by UV does not have any observable effect is consistent with our previous observations that only one of the two DNA strands is expressible in the process of transformation (J. Marmur, D. Lane and P. Doty, unpublished results).

2. *Restoration of biological activity by photoreactivating (PR) enzyme.* Rupert, '60 has shown that the biological activity of UV inactivated transforming DNA from *H. influenzae* can in part be restored by incubation with an enzyme from bakers'

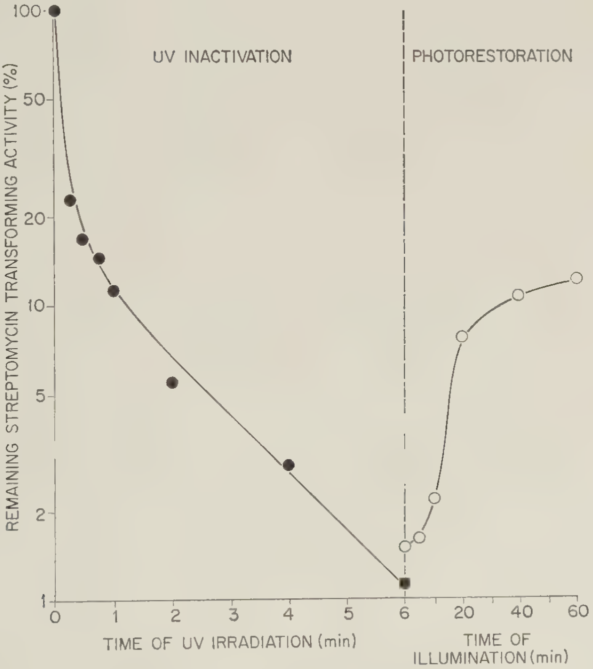


Fig. 18 Effect of UV light and photoreactivating enzyme on the streptomycin-transforming activity of *D. pneumoniae* DNA. *D. pneumoniae* DNA was irradiated with the same UV lamp and at the same light intensity as in figure 17. In a separate experiment, DNA which had been irradiated to a level of approximately 1% remaining streptomycin transforming activity, was incubated with an extract from bakers' yeast (1 mg/ml) at 37°C. at a distance of 2 cm from a Sylvania 15 watt "cool white" (F15T8-CW) lamp. After varying periods of illumination, aliquots were removed and assayed for their ability to transform with respect to the streptomycin marker. This curve is plotted to the right of the dashed vertical line.

yeast in the presence of light. This enzyme, as well as an extract from *E. coli* (Rebeyrotte and Latarjet, '60), also restores the transforming activity of UV irradiated DNA from *D. pneumoniae*. Figure 18 shows that when the DNA has been irradiated to a level of approximately 1% remaining activity, the ability to transform sensitive cells to streptomycin resistance can be restored to a level approximately 10% of the initial value when incubated with the PR enzyme in the presence of visible light.

Using this enzyme, it was shown that the UV induced, denaturation-resistant cross-linking of the DNA strands could be restored to a denaturation-sensitive state (Marmur and Grossman, '61). The method employed to follow the changes from the denaturation resistant to the sensitive state was by the use of the *E. coli* phosphodiesterase which acts only on denatured DNA.

DISCUSSION

Ultraviolet irradiation of DNA results in a complex series of effects which can be recognized and studied at chemical, physical chemical and biological levels. The initial effects following the absorption of a photon (which causes radiation damage) is most likely a chemical alteration of the pyrimidine residues (Shugar, '60). This type of lesion is most likely followed by a weakening of the DNA structure resulting from an interference in normal hydrogen bonding; experimental evidence supporting this has been presented. Other effects have been noted. Cross-linking of the DNA strands and backbone scission have also been detected, the latter as a result of relatively high UV doses. Cross-linking between the complementary strands resulting from the dimerization of specific base pairs that are not normally opposite one another would most likely require the initial local denaturation or distortion of the molecule. Cross-linking may also take place by dimer formation *within denatured* DNA strands. It is not known at the present time which of these types of damage (or of some other type) is responsible for the UV effects on gene action or the induction of mutations. The inactivation of organisms possessing single stranded DNA, e.g., φX 174, could not readily be explained by local denatura-
tion nor by cross-linking between *comp*
mentary strands.

The formation of dimers of thymine
a result of UV irradiation (Beukers a
Berends, '60; Wang, '60, '61) would app
at present to be the most promising
proach for explaining the biological effe
of UV irradiation. Cross-linking by dim
formation would most likely impair DN
replication and proper base pairing. Ho
ever, the cross-linking, if it is to be
voked to explain the UV irradiation i
pairment of gene action, would have
prevent *in vivo* strand separation only
the regions of the UV induced lesions. Th
would be necessary to explain the diff
ential inactivation of linked gene
markers residing on the same DNA mo
cule. The observed differential sensitivit
might result from the presence and/
arrangement of specific base residues co
ducive to dimer formation and the subs
quent impairment of expression of th
region.

In seeking an explanation for the dosag
response curve for the UV inactivation
transforming activity, it has been fou
that pretreatment of the DNA (sonic
HNO_2) renders the remaining activity mo
resistant to UV inactivation. The mul
component nature of the inactivation cur
for native DNA might result from any
all of the following factors: (1) a heter
geneity in size of the molecules carryi
the marker under investigation; (2) t
possibility of a diminution in the lifeti
of the UV induced excitation with i
creased accumulation of lesions surroun
ing the marker (L. S. Lerman, person
communication); and (3) the probabili
of recombination rescue by the genome
the recipient cells of the damaged are
linked to the marker which becomes pr
gressively smaller as the damages a
proach the marker region itself.

SUMMARY

When DNA is irradiated in solution wi
UV light at 254 mμ, loss of transformi
activity and changes in the physical che
ical properties of the DNA can be detecte
The loss of biological activity, studied wi
DNA isolated from *D. pneumoniae* and
subtilis, depends on the genetic mark
under investigation as well as on its si

on the molecular weight of the DNA g irradiated.

hen exposed to higher dose levels, na- DNA is both partially degraded and kened in structure. Cross-linking of complementary strands has been de- d. The UV induced physical chemical ations have been detected by the lower- of the denaturation temperature, a in sedimentation coefficient after de- ration, a decrease in viscosity, an in- se in buoyant density in a CsCl gradi- a cross-linking of DNA strands and an ease in the pH at which the molecules ture. Most of the physical chemical nges are accentuated by a higher ade- plus thymine content of the DNA and he substitution of 5-bromouracil for nine. Denatured and native DNA be- differently when exposed to the same at the same wavelength. As in the gical experiments, for the same dose ls, the smaller the molecular weight of DNA, the less the physical chemical age. Both the biological and the vis- ty changes induced by UV show no gen effect. The photoreactivating en- e from bakers' yeast reactivates UV- ted transforming DNA in the presence isible light; the reactivation might be in part to the disruption of the cross- s induced by UV.

ACKNOWLEDGMENTS

he authors would like to thank Drs. C. ildkraut, K. L. Wierzchowski, S. Y. ng, L. Grossman and D. M. Green for r valuable suggestions and discussions. y also wish to thank Mr. M. L. Coval Dr. F. Bergmann for their aid in the toreactivation experiments.

OPEN DISCUSSION

HAIRMAN ALEXANDER[6]: I suggest a ful way of organizing the discussion ht be to leave any questions concern- photoreactivation until after the sec- paper, and talk over first the many er aspects raised by Dr. Marmur.

. SETLOW[7]: You mentioned that UV sitivity was related to marker size. ve you any other criterion for marker e?

MARMUR: Dr. Hotchkiss and his associ- s have developed an interesting method of estimating the marker size of the genetic units involved in the transformation of *Diplococcus pneumoniae* to sulfanilamide resistance or maltose utilization by exposing the transforming DNA to elevated temperatures. They have found that by heating the DNA below the melting temperature, the rate of inactivation of the transforming activity can be related to the size of the genetic marker. This conclusion was supported by genetic experiments. If you heat DNA at a temperature below the T_m, depurination is likely to occur. The larger the size of the marker on the molecule, the more easily depurinated it is likely to be. This characteristic ties in with the idea that it is a larger-sized marker.

WIERZCHOWSKI[8]: The evidence presented by Dr. Marmur for a cross-link between the strands in DNA duplex is very likely the evidence in favor of thymine-dimer formation between two thymine residues located in opposite strands of DNA molecule. But there is also a good deal of evidence from studies on the synthetic polynucleotide systems composed of cytidylic acid and purine nucleotides that upon UV-irradiation, cytosine takes up a water molecule across 5,6 double bond.

I would like to make some comments as far as it concerns a possible relationship between both reactions.

Our photochemical studies on systems like poly U-poly A complex (1:1) and poly C-poly I complex (1:1) revealed that UV-irradiation of these complexes in water solution causes the stepwise dissociation of the double-helical structure as a result of photohydration of uracil and cytosine bases. Moreover, when you irradiate poly U alone you observe two types of photochemical reactions. One of them, heat irreversible, presumably involves photodimerization of adjacent uracil bases. This follows from our studies on uracil di- and tri-nucleotides, which have shown that photodimirization is connected with the presence of neighboring uracil residues along the polynucleotide chain, as well as from the data on irradiation of poly-U in dry state and simple di-keto pyrimidine derivatives in frozen state. The other reaction which you observe is heat reversible photohydration of uracil bases. But in the complex poly U-poly A (1:1), until some 30% of uracil residues have been reacted, only the reversible reaction takes place which means that in this double-helical molecule there is no possibility for photodimerization reaction. Inspection of the model of DNA shows that the same conclusion holds with respect to thymine bases in DNA. The mutual position of neighboring thymine residues in this same as well as in opposite strands does not allow them to dimerize. One can realize, however, that upon UV irradiation of DNA in a water solution the first photochemical process is photohydration of cytosine bases with resulting dissociation of hydrogen bonds between reacted complementary C and G residues. This local denaturation of the DNA structure makes possible the proper adjustment of thymine bases in surrounding of damaged photochemically region and their subsequent photo-dimerization.

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[6] P. Alexander, Chester Beatty Research Institute, London.

[7] J. Setlow, Oak Ridge National Laboratory.

[8] K. L. Wierzchowski, Harvard University.

This sequence of photochemical events could probably be demonstrated by studies on the dependence of inactivation of transforming DNA or of amount of cross-link involved on intensity of UV-beam. The comparably high specific reaction rate constant for the elimination of water from the photohydrated cytosine bases leads at low intensity of UV-light to a photochemical equilibrium whose position depends on used intensity. For this same total UV-dose delivered at lower dose-rate one would expect lesser degree of inactivation or amount of cross-linked molecules of DNA.

Kaplan[9] : I was going to ask Dr. Marmur to elaborate a bit on whether the thymines in two opposite strands of DNA can really see each other to the extent of forming a dimer. I take it your evidence indicated some non-dissociation under the conditions in which you would expect denaturation, but you have no direct evidence for denaturation as such. I wonder if the two thymines can actually see each other?

Marmur: We do not actually have any chemical evidence in our experiments that the cross-linking of the complementary DNA strands is the direct result of the formation of dimers of thymine. However, the work of Wacker and his associates well as of Beukers and Berends is pertine They have irradiated DNA and, after h drolysis, identified material that behav chromatographically and in other respec similarly to dimers of thymine.

Since we have demonstrated cross-lin ing between strands of UV irradiated DN (Marmur and Grossman, '61), it is di cult to imagine how this can be explain by the formation of thymine dimers unle the irradiation causes some initial den tured regions. We believe that our resu do show that some denaturation or wea ening of the irradiated DNA molecule c indeed take place.

Elkind[10] : I would like to ask you fir is there any indication that the amount cross-linking is related to the amount thymine in the DNA? Secondly, do y have any information about the effects the halogenated pyrimidines on the like hood of cross-linking?

Marmur: We do have some prelimina experiments indicating that, in general, th higher the adenine-plus-thymine content the DNA, the more likely it is to form cros links between the complementary DN strands on irradiation. Cross-linking h also been demonstrated for DNA isolate from irradiated microorganisms.

We have not yet studied cross-linking DNA containing halogenated pyrimidine I believe that the inactivation of such DN is different than that of normal DNA. F instance, the introduction of halogenate derivatives into DNA causes it to be mor susceptible to irradiation-induced bac bone breakage.

Wolff[11] : May I get something clea Are you saying that the mutagenic eve as measured by marker loss, is cross-lin age of DNA so that at the place where th mutagenic event has occurred DNA doesn open up and renature? Or do you think th mutagenic event might be something else

Marmur: When DNA is irradiated, w detect some cross-linking between th strands. However, other types of dama can be detected that probably contribu to the loss of biological activity. Which

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[9] H. S. Kaplan, Stanford University.

[10] M. M. Elkind, National Cancer Institut Bethesda.

[11] S. Wolff, Oak Ridge National Laboratory.

e types of damage is responsible for a
ation is unknown at the present time.
conceivable that the formation of
s-links between the strands prevents
complete separation and normal func-
s in replication and also may give rise
bnormal base pairing which is event-
recognized as a mutation. The lat-
effect might also take place if the
erization occurs *within* the DNA
nd.

A CHANCE[12] : I should like to clear up
int of confusion. In your early sonica-
experiments you mentioned that you
up with shorter molecules with the
ker intact. Is there never breakage
in the marker so that you get inac-
tion or loss of the marker?

ARMUR: Sonic treatment itself of DNA
ses a drop in its transforming activity.
s, in the UV inactivation curves of the
cally treated transforming DNA sam-
, we have normalized the results by
ng the per cent transformation of
n of the non UV-irradiated samples to

OVELLI[13] : Just on that last point, in
e shorter molecules, if you sonically
t the DNA, do you get the same T_m?

ARMUR: The melting temperature, or
of sonically treated and non-treated
A is essentially identical. This has
n observed for DNA of bacterial and of
mal origin.

ENDELSOHN[14] : Have you tested the
sphodiesterase on single-stranded UV-
diated DNA?

ARMUR: Irradiating it first in the
gle-stranded phase?

ENDELSOHN: Yes.

ARMUR: No. We haven't done that.

. B. SETLOW[15] : We studied the quan-
n yield of dimer formation by 2650 Å
iation. It is ~ 0.5.

should point out, apropos of thymine-
er reaction, that the action spectra for
two reactions—one, the production of
imer from thymine and second, the de-
ction of the dimer that is formed—are
te different from one another. For ex-
ple, when one forms this dimer, the
orption spectrum shows no maximum
the neighborhood of 260 mμ but only
ws end absorption at low wave lengths.

Most of you are familiar with the shape of the absorption spectrum of thymine, which on a scale of this type has a maximum in the neighborhood of 265 mμ. When one irradiates thymine—and this is the work of Beukers and Berends—one finds primarily that the product that is formed has no absorption in this region. The product is easy to see, in a sense, because on a relative scale the product has an absorption spectrum of the indicated form.

If one irradiates the product which contains no resonating rings, one obtains thymine. My point is that the action spectrum for the reversal form product to thymine looks like the absorption spectrum of the product as one would expect, and that the short wave lengths, 220 and 230 mμ are most efficient in reversing the reaction and presumably the long wave lengths are efficient in causing the dimerization of thymine.

Therefore this is an experimental way of getting at the two parts of the reaction. One wave length makes product, presumably thymine dimers; and another wave length splits the dimer into thymines. This is the way of getting at the two steps. Presumably one could find out whether one really has thymine dimers by doing the experiment in this fashion.

WINKLER[16] : We did some cross-reactivation (CR) experiments with monochromatic UV to find out whether the UV-induced dimerization of thymine is responsible for some biological UV effects. The ratio of the extinction-coefficients of thymine monomer to dimer is about 43:1 at 265 mμ and 1.3:1 at 235 mμ. We thought, if the final part of a typical CR-curve is due to an equilibrium between dimerization and monomerization then we can expect that the fraction of markers rescued in a 235 mμ experiment is larger than in a 265 mμ. We could not solve our problem because we got some unexpected UV effects in the phage protein at 235 mμ: many phages show even at low doses tail-lesions and release the DNA. Therefore the fraction of markers rescued after 235 mμ irradiation is much less than in a 265 experiment. This work was done together with Dr. Harold Johns.

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[12] L. E. LaChance, U. S. Department of Agriculture, Kerrville.

[13] G. D. Novelli, Oak Ridge National Laboratory.

[14] M. L. Mendelsohn, University of Pennsylvania.

[15] R. B. Setlow, Oak Ridge National Laboratory.

[16] U. Winkler, California Institute of Technology.

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epair of Ultraviolet Damage in Cellular DNA[1]

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The preceding paper in this Symposium
armur *et al.*, '61) is the natural intro-
ction to the present topic. As Dr. Mar-
r has shown, ultraviolet (UV) radiation
duces photochemical changes in deoxy-
onucleic acid (DNA) which modify its
ysical-chemical properties. Like most
anges that can be made in the compo-
nts of an intricate mechanism, these
erations are functionally harmful and
ult in lowered biological activity as
dged by the diminished ability to pro-
ce genetic transformation of bacteria.
en if nothing else in a cell were dam-
ed, we might expect such changes to
ve observable metabolic consequences.
This expectation is supported by the ac-
n spectra for many UV effects in orga-
ms, which resemble the absorption
ctra of nucleic acids, suggesting that
se substances actually absorb the ef-
tive radiation (see Zelle and Hollaen-
, '55 and Setlow, '57 for examples).
hough UV induces detrimental changes
purified viral ribonucleic acid (RNA)
shown by its decreased infectivity (Sie-
, Ginoza, and Wildman, '57), particular
ention is drawn to DNA by its uniquely
ntral position in the cellular organiza-
n. Moreover, many of the most striking
nsequences of UV irradiation appear to
olve nuclear or genetic processes rather
n other parts of the cellular metabo-
n. Therefore, it seems likely that DNA
mage constitutes at least a noticeable,
not a major, part of UV action on organ-
ns. We may inquire about the response
the biological system to this injury.

Cells have been confronted with UV
liation in the form of natural sunlight
oughout the evolution of life on earth,
d the intensity of this radiation today
enough to present a significant challenge
cellular DNA. An hour's exposure of
nsforming DNA to noon sunlight in a quartz container (at sea level and latitude 40° between April and August) results in an inactivation equivalent to about 2000–4000 ergs/mm^2 of 254 mμ radiation. This dose would reduce the survival of *Escherichia coli* B/r by 3 to 4 log units. T2 bacteriophage lose about 1 log unit of infective titer for every five minutes exposure under the same circumstances indicating a similar order of dose equivalence. This inactivation is evidently caused by the shortest wave lengths penetrating the earth's atmosphere (those near 300 mμ) (Sanderson and Hurlbut, '55), since it is considerably reduced by thin pyrex glass, is very sensitive to the altitude of the sun, is eliminated entirely by a filter (Corning No. 7380) that cuts off wave lengths below 350 mμ, and is little affected by a nickel sulfate filter that is fairly transparent below 320 mμ, but eliminates radiation between 340 and 450 mμ. Thus it corresponds to the bactericidal and mutagenic actions of sunlight known for many years (Zelle and Hollaender, '55). Faced with this radiation assault on their genetic material over geologic periods of time, cells might be expected to evolve mechanisms for counteracting it.

Almost any one of the many factors modifying the effects of UV light on a cell—the post-irradiation temperature, composition of the medium, state of illumination with other wave lengths, previous treatments of the cells—might indicate a recovery mechanism which was being stimulated or inhibited by the treatment, but the situation particularly favors search for such a mechanism in photoreactivation. (See Jagger, '58 for a review.)

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Here the decrease in effectiveness of a UV dose produced by later illumination at longer wave lengths is usually large, the phenomenon is observed in a great variety of organisms and it can easily be turned on and off—merely by applying or withholding light—without any great disturbance of the cellular system as a whole.

Repair of irradiated DNA in vitro

Transforming DNA inactivated by short wave lengths of sunlight or by 254 mμ radiation can be restored to activity by photoenzymes from photoreactivable cells (Rupert, Goodgal, and Herriott, '58; Rupert, '60a, '60b). When these enzymes are mixed with the damaged DNA and exposed to light, progressive recovery occurs up to a high level, the maximum corresponding to erasure of about 90% of the UV dose. Recovery ceases when the light is interrupted, and resumes at the same rate at which it left off when illumination is reapplied.

The reactivating light, lying midway between 300 and 400 mμ, is well supplied by sunlight, so that the entire process of inactivation and recovery can be carried out without artificial sources of radiation of any kind. Transforming DNA exposed to the sun in a quartz flask until its activity is reduced by 2–3 log units can be brought back to about 10% of the unirradiated activity by simply adding a small amount of photoreactivating enzyme and continuing the exposure in the same quartz flask (Rupert, '60b). Adding the enzyme at the start of exposure markedly lowers the rate of inactivation, even though the optical transparency of the solution for $\lambda > 290$ mμ is high.

This enzyme activity has been found in the two photoreactivable species (*E. coli* and *Saccharomyces cerevisiae*) which have been examined for it, but it is not found by the same techniques in the non-photoreactivable cell of *Hemophilus influenzae* from which the transforming DNA was derived (Goodgal *et al.*, '57). It therefore offers a possible explanation for the cellular recovery observed in photoreactivation.

It is relatively easy to show that transforming DNA is damaged inside the cell by mercury arc radiation (S. H. Goodgal, unpublished) or by sunlight simply b radiating the intact cells before extrac and assaying activity of their DNA. analogous direct test for repair of damage inside the cells—by photore vating irradiated cells before the extrac and showing reversal of the intracellul occurring damage—cannot yet be m because the species commonly used transformation (*H. influenzae, Diploco pneumoniae, Bacillus subtilis*) all ph reactivate very poorly if at all (Jag '58). For this demonstration, the enz system must be used indirectly as scribed below.

The enzymes obtained from *E. coli* from yeast produce the same degree recovery in a sample of irradiated tr forming DNA whether used singly or after the other. Consequently we pres they repair the same kind of damage. enzyme from baker's yeast has been a lyzed in most detail.

The yeast enzyme combines with irradiated DNA in the dark, as can shown in several ways. During ultra trifugation the enzyme activity sedim rather slowly and can be recovered f the upper portions of a preparative cen fuge tube after several hours at 100, × *g*. Under the same circumstances D will sediment to the bottom of the t and in a mixture of the two each beha in its characteristic fashion providing DNA is unirradiated. However, if DNA has been UV-irradiated, the enz activity follows it to the bottom of tube (Rupert, '60b).

A similar demonstration of the s fact is furnished by gel filtration (Por '60). Unirradiated DNA and enzyme pas together through a column of Sepha G-75 (manufactured by Pharmacia, U sala, Sweden) are slightly separated. radiated DNA and enzyme, on the o hand, come through together.

The yeast enzyme is stabilized by c bination with its substrate, so that the of inactivation by heat and heavy me is markedly reduced in the presence irradiated DNA (table 1). Unirradia DNA does not stabilize the enzyme. protection against heat is independen irradiated DNA concentration when

TABLE 1

Protection of photoreactivating enzyme from heat inactivation by irradiated DNA

Mixtures of yeast photoreactivating enzyme with 0.15 M saline or with irradiated or unirradiated non-transforming DNA in saline were made up in duplicate pairs. One member of each pair was heated to 63°C. for 1 minute and cooled. Ultraviolet irradiated transforming DNA was added, the mixtures illuminated and rates of photoreactivation (in arbitrary units) determined for each (Rupert, '60a). Residual activity of the heated enzyme is expressed as a fraction of that in the duplicate, unheated control. The corresponding half life at 63°C. is computed on the basis of exponential inactivation, as shown in separate tests. The UV dose and the concentrations of DNA and enzyme were selected to provide maximum protection.

Non-transforming DNA present	Min. at 63°C.	Recovery rate	Residual activity	Half life
				(seconds)
None	0	1.06	0.35	40
	1	0.37		
Unirradiated	0	0.96	0.30	36
	1	0.28		
Irradiated	0	0.92	0.77	168
	1	0.71		

sufficiently high, presumably because
the enzyme is combined with substrate.
ther abruptly at lower concentrations
protection begins to diminish with
ution of the irradiated DNA, presum-
y because part of the enzyme is now
uncombined.

We therefore have evidence for the
al first step of an enzymatic reaction:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES,$$

ere the substrate S represents UV dam-
in DNA.

This complex of a UV lesion and the
otoreactivating enzyme is dissociated by
ht. If sufficient illumination is applied
a mixture of enzyme and irradiated
A before centrifugation enzyme activ-
is again left behind in the top of the
e as it is with unirradiated DNA. Sim-
ly the amount of heat protection de-
ases with exposure to light before heat-
. Under conditions where the amount
heat protection depends on DNA con-
tration the decay is approximately ex-
ential with illumination time. Since
air occurs only during this illumination,
have

$$ES \xrightarrow{k_3} E + P,$$

ere the product P is the repaired struc-
, and the rate constant k_3 is zero in
dark.

These two steps add up to a conventional Michaelis-Menten reaction scheme, and a kinetic analysis agrees, showing that the dependence of recovery rate on irradiated DNA concentration is that predicted by Michaelis-Menten kinetics. The rate constant k_3 increases with increasing light intensity.

As might be expected, competitive inhibition of the reaction can be observed when irradiated non-transforming DNA is added to the reaction mixture (Rupert, '58). This inhibitory power can be eliminated by allowing the enzyme to act on the competing DNA in the light—so that the competing UV lesions are repaired—before mixing with irradiated transforming DNA to test for the inhibition.

These characteristics provide the means for detecting photoreactivable UV lesions in any sample of DNA without regard to its biological activity, the inhibition and heat protection phenomena furnishing a particularly happy combination for this purpose. If an unknown sample is added to a reaction mixture of photoreactivating enzyme and UV-irradiated transforming DNA, the presence of UV lesions will be indicated by competitive inhibition; i.e., the recovery rate in the mixture will be lower than in an appropriate control. If the same material, diluted until its competitive inhibition is negligible, is mixed with photoreactivating enzyme in the dark and heated briefly, the presence of UV lesions will be indicated by partial protection of the enzyme from inactivation; i.e., when the enzyme is tested for activity the recovery rate of irradiated transforming DNA will be higher than in a control. Both effects can be eliminated by preilluminating the material under test in the presence of the enzyme, but not by incubating with it in the dark. Such a specific combination of characteristics minimizes the danger of misleading artifacts when studying relatively impure materials from cells.

The heat-protection effect was found later than the competitive inhibition, consequently in a number of studies involving purified DNA we employed only the inhibition—checking in each case that it could be eliminated by preillumination with the enzyme but not by preincubation in the dark. According to this test photoreactivable UV damage occurs in all normal DNA's examined: those from *H. influenzae*, *E. coli*, T2 and ϕX-174 bacteriophage (supplied by Dr. R. L. Sinsheimer) and calf thymus tissue as well as Kornberg's enzymatically synthesized material (Bessman *et al.*, '58) using natural DNA as primer. This repairable damage thus occurs in both single- and double-stranded DNA from both photoreactivable and nonphotoreactivable species. The amounts of damage developed in all are comparable for the same UV dose.

Abnormal DNA's may be different. *E. coli* DNA with about 35% of its thymine substituted by 5-bromouracil, which was studied in collaboration with Dr. Sheldon Greer of Columbia University furnishes a particularly interesting example. After irradiation this material gives the same amount of competitive inhibition as control DNA from the same strain of cells grown with thymine, while neither the control nor the 5-bromouracil DNA's inhibit when unirradiated, nor after exposure to the reactivating light in the absence of enzyme. As expected, the lesions in the irradiated normal control can be repaired by pretreatment with the enzyme in the light, eliminating the observed inhibition. However, those in the 5-bromouracil DNA cannot, the inhibitory power actually increasing appreciably during pretreatment. This material is thus n photoreactivable like the cells from wh it was taken (Greer, '60). A similar ef has been observed when 5-bromouraci incorporated into bacteriophage (St *et al.*, '61). Such parallel behavior of *in vivo* and *in vitro* photoreactivation tems upon addition of 5-bromouracil the DNA supports the idea that cellu photorecovery involves DNA repair.

Repair of irradiated DNA in vivo

More direct evidence is provided by amining the DNA from normal irradia cells, both before and after their phot activation. We are not limited in this c to transformable species, but can emp any convenient organism. It is not e necessary to purify the DNA before t ing—since irradiated RNA does not in fere in this system—but the crude lysate must be deproteinized and the and ionic strength adjusted by dial before testing.

Figure 1 shows the usual photoreact tion of a culture of *E. coli* B/r produ by illuminating the cells after irradiat to 1% survival (2150 ergs/mm² 254

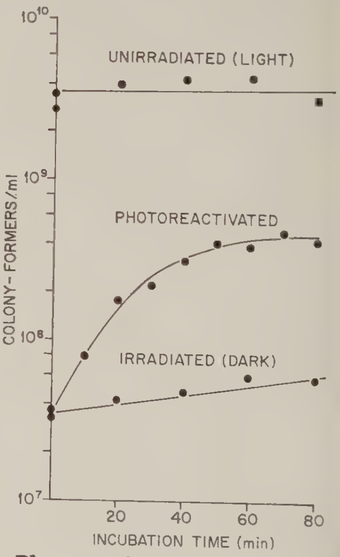
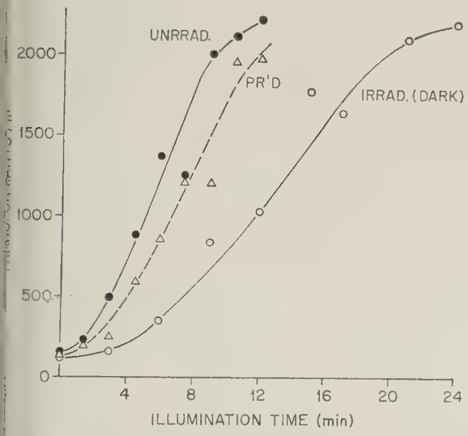


Fig. 1 Photoreactivation of irradiated Es *richia coli* B/r (2150 ergs/mm² 254 mμ ra tion) with unirradiated and dark-incubated trols. Cells were grown to stationary phase M-9 glucose-ammonium-phosphate medium, suspended in saline and incubated with aera before irradiation.

iation). An aliquot of the unirradiated ture similarly illuminated and of the adiated cells incubated in the dark serve controls. At the conclusion of this treat- nt identical volumes of the three ali- ots were lysed with desoxycholate and monium hydroxide, the mixtures neu- lized and shaken with chloroform-oc- hol in the presence of 2 M NaCl and aqueous phases dialyzed against buf- ed saline. Optical densities at 260 mμ owed the same concentration of cellular terial in each preparation. These prepa- ions were then tested for the competi- e inhibitory and heat-protective proper- s with the results shown in figures 2 d 3.

Lysates of unirradiated cells have no ect by either test. The recovery curves rresponding to these preparations are ntical with those obtained when no cell ate is added, showing that no UV lesions e present in the unirradiated cellular NA. Lysates of the irradiated cells incu- ted in the dark show both competitive hibition and heat protection. Both ef- ts can be markedly reduced or elimi- ted by pretreating the lysates with the zyme in the light before testing, showing that photoreactivable UV damage is present in the irradiated cellular DNA. Lysates of photoreactivated cells show these effects only at a much lower level, implying a smaller concentration of photoreactivable lesions in the cell population. Such behavior may be taken as evidence that most of this damage has been removed by intracellular photoreactivation.



ig. 2 Presence and disappearance of photo- ctivable UV lesions in DNA of cells shown in re 1, indicated by competitive inhibition of nsforming DNA repair. Curve marked "un- adiated" represents uninhibited recovery in the sence of deproteinized lysate of unirradiated s. "Irradiated" represents inhibited recovery the presence of lysate of irradiated, non-photo- ctivated cells. "Photoreactivated" represents a -inhibited recovery in the presence of lysate photoreactivated cells.

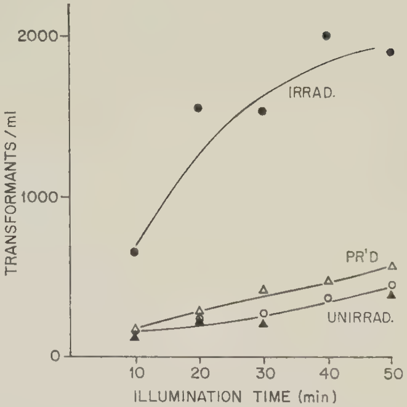


Fig. 3 Presence and disappearance of photoreactivable UV lesions in DNA of cells shown in figure 1, indicated by heat protection of photoreactivating enzyme. Each curve shows recovery of irradiated transforming DNA produced by a heated sample of enzyme. Curve marked "unirradiated" represents enzyme heated with undiluted (●) or diluted (○) lysate of unirradiated cells, giving no protection. "Irradiated" represents enzyme protected from heat inactivation by diluted lysate of irradiated, non-photoreactivated cells. "Photoreactivated" represents enzyme only slightly protected from heat inactivation by diluted lysate of photoreactivated cells.

DNA repair in lightly irradiated cells

The story would be clearer if we knew the location of the remaining damage after maximal photoreactivation. Since even after photorecovery only 1 cell out of 10 could form a colony in these experiments, the observed appearance and removal of UV lesions involves essentially non-colony-forming cells. It makes a difference in the meaning of the above results whether the residual damage is thinly sprinkled in the cells which do not survive as colony-forming units (as seems likely), or whether it is by any chance concentrated in the small minority which do.

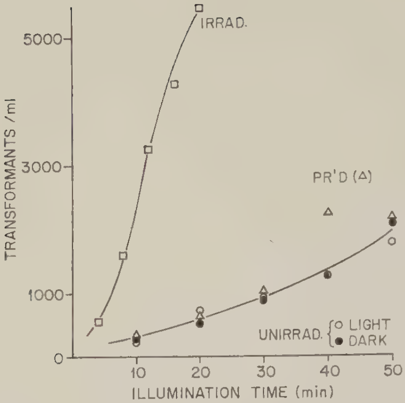


Fig. 4 Presence and disappearance of UV lesions in DNA of lightly irradiated *E. coli* B/r (350 ergs/mm² 254 mμ radiation). Each curve shows recovery of irradiated transforming DNA produced by heated enzyme. "Unirradiated" represents enzyme unprotected from inactivation during heating with lysate of unirradiated cells which had been incubated either light (○) or dark (●). "Irradiated" represents enzyme protected from heat inactivation by lysate of irradiated cells incubated in the dark. "Photoreactivated" represents enzyme unprotected from heat inactivation by lysate of irradiated cells incubated light.

To clarify this point the test was repeated at a much lower UV dose—350 ergs/mm², giving a survival of better than 75% even with no photoreactivation. No attempt was made to follow photorecovery by viable count at this high survival, but light was simply applied for 20 minutes—about four-tenths the time required for maximum recovery with the 6-fold larger UV dose employed before. The results of applying the heat protection test for UV lesions to the cell lysates, shown in figure 4, indicate a very high fraction of the UV lesions were removed by intracellular photoreactivation, although in undiluted lysates they could still be detected. This seems to say that essentially all the photoreactivable lesions in DNA of viable cells can be removed intracellularly.

The sensitivity of the heat protection test would permit detection of UV damage at still lower doses. It should be possible therefore to study "photoreactivation" in cell populations with close to 100% survival at all stages of the experiment. This low dose level should represent a more "normal" operation of the recovery mechanism than the customary higher do
which damage most of the cells bey
repair.

The small rise in viable count of
irradiated cells incubated in the da
which appears in figure 1, may repres
the previously known recovery of irra
ated cells held in liquid media before pl
ing (Zelle and Hollaender, '55), althou
it did not appear in all repetitions of
experiment. Lysates of irradiated B/r ce
held at ice temperatures in saline for 9
150 minutes showed higher concentratio
of UV lesions than lysates of the sa
cells incubated in the dark, suggesti
that some sort of repair occurs even in
absence of photoreactivation. Howev
since these experiments were carried
at the 1% survival levels, most of
DNA coming from non-reactivable cells
is not yet certain that this decrease in
concentration of lesions is related to i
proved survival.

CONCLUSIONS

The story as it now stands can be su
marized as follows: Enzymes exist in ph
toreactivable cells which will repair
radiation damage to transforming DN
in vitro. By study of the *in vitro* enzyma
reaction it can be inferred that similar
pair is effected in other natural DN
lacking recognized biological activity. T
broad outlines of the enzymatic mech
nism seem well established, but one h
yet to fill in any chemical details.

Something happens to the UV dama
of cellular DNA during photoreactivati
of cells so that it can no longer be detect
in cell lysates. The disappearance is co
sistent with its repair, although in the
sence of a recognized biological activity
the extracted DNA, it cannot be certa
that this material has returned to its ori
nal state.

The DNA damage repairable by
photoreactivating enzyme *in vitro* is p
duced by short wave lengths of natu
sunlight, and can be repaired by this
zyme at the same time it is being p
duced, utilizing the longer wave leng
of sunlight to drive the reaction.

The above facts seem fairly clear. Fr
circumstantial evidence the following

onal points might be surmised, subject
urther argument and investigation:
. Photoreactivable UV lesions in DNA
se a significant fraction of the total
cts of UV on cells. Their repair is
ntial if these effects are to be pre-
ted. These lesions therefore correspond
t least in part — to the radiation-in-
ed "poison" sometimes invoked to ac-
nt for UV phenomena in organisms
., Novick and Szilard, '49).
. Photoreactivation is one of the mech-
sms evolved by cells to repair the dam-
produced by sunlight in their DNA.
ause it is so widespread among living
gs and because it could in principle
ur with such a low level of cellular
anization — requiring only a suitable
alyst to favor the photoreversal of a
tochemical change — it could be con-
ered a primitive mechanism, evolving
rly early in the history of life on earth.
. Other more sophisticated mecha-
ms probably exist to perform the same
ction and may be responsible for some
the factors modifying the effects of
radiation on cells (see, for example,
y, '59).
Vith the ability to detect a primary UV
nage in cells we move into a new era
radiation biology. An initial biochemi-
lesion can now be observed apart from
metabolic consequences, and the rela-
nship between the two studied in the
ural sequence of cause and effect. We
refore gain increased resolution of the
nplex events associated with UV injury
d move a step nearer the description of
iation processes in chemical terms.

OPEN DISCUSSION

R. B. Setlow[2]: Could you tell us some-
ng about the competing ability of UV-
diated DNA that has been heated above
melting point?

Rupert: Yes. It does just about the
ne as undenatured DNA. So does
-174 DNA.

R. B. Setlow: Have you UV-irradiated
A at room temperature and taken it
ve the melting point and down?

Rupert: It doesn't make any difference
ether you denature it after you irradiate
before—you get the same answer. The
same thing applies to acid denaturization or alkali denaturization. The UV lesions will survive pH 2.5 overnight or pH 12.5 overnight at room temperature, or 100°C. for 40 minutes. They are fairly stable chemical entities.

Heinmets[3]: Have you tried to identify the primary site of photon absorption? This need not be the same when different spectral regions are used for radiations.

Rupert: The final damage produced by 300 mμ and by 254 mμ radiation seems to be the same as far as the enzyme is concerned. It apparently recognizes the damage as its substrate in both cases and goes to work on it the same way.

Heinmets: What kind of photochemical reaction is produced?

Rupert: We don't know any photochemistry.

Heinmets: In complex biological structures we may have a photochemical reaction which produces molecular alterations in the system; this may lead into secondary interactions, etc. It is strange and disturbing when molecular processes are referred to as "lesions."

Rupert: I must have some grammatically convenient word for the UV damage in DNA in order to talk about it. When we know its real chemical nature, we will call it that. In the meantime the term "UV lesion" (that is, "UV injury") fills the need. By "lesion" I just mean the localized photochemical alteration of DNA which affects its biological function and which this enzyme recognizes as its substrate and will repair.

Kaplan[4]: One might infer that the bromouracil-DNA might possibly combine with your enzyme, but not be subjected to photolysis by light. Have you tried your sedimentation experiments over again with bromouracil?

Rupert: No, and we haven't tried the heat-protection test. These things certainly should be done.

Hutchinson[5]: Have you any information on reactions between your enzyme and the irradiated nucleotides or unirradiated compounds of any kind?

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[2] R. B. Setlow, Oak Ridge National Laboratory.

[3] F. Heinmets, Quartermaster Research and Engineering Center, Natick.

[4] H. S. Kaplan, Stanford University.

[5] F. Hutchinson, Yale University.

Rupert: No single nucleotide seems to work, irradiated or not. Up to several per cent of the phosphate ester bonds can be digested by pancreatic DNase without noticeable effect on the competitive inhibition. But DNA digested to the limit won't inhibit after irradiation. This of course immediately complicates the problem of isolating a chemically pure substrate. One can't just irradiate DNA, chew it up thoroughly with DNase, fractionate the small molecules and determine which fraction works. It isn't that simple. What probably could be done but hasn't, is to digest as far as possible while still preserving some inhibition, and then try to characterize the simplest active fraction by determining what bases are present, and so on.

As far as the large molecules are concerned, neither polyribonucleotides nor RNA do anything at all. The poly deoxy-AT does nothing we can see. The poly deoxy-GC gives a very weak effect, corresponding to about a 6th or a 7th as much UV damage for the same UV dose as the same amount or normal DNA (measured by competitive inhibition). The photoreactivable UV damage is apparently there after irradiation. It inhibits and can be wiped out by letting the enzyme act on it, but it is present in abnormally small amounts. What this means, I don't know.

Kelly[6] : Have you observed any competitive inhibition with DNA that has been irradiated with ionizing radiation?

Rupert: Dr. Gray and his coworkers are going to help us try to do that experiment.

Krieg[7] : You mentioned that the 5-bromouracil-containing DNA, when irradiated, received lesions which compete for the enzyme, but that these lesions cannot be eliminated by the enzyme. Does the same situation exist with irradiated denatured DNA? Have you looked to see whether the lesions there can be removed by the enzyme?

Rupert: Yes. And they can be.

Jagger[8] : Is there any evidence that your enzyme combines with RNA?

Rupert: Irradiated RNA gives no more competitive inhibition than unirradiated.
For a given UV dose the number of ph
reactivable UV lesions per unit of mate
must be below 1% of what it is in
DNA, and actually there is no evidenc
any at all. It looks like this enzyme sim
works on DNA and not on RNA, altho
I don't know why in view of the fact
bases are essentially the same and
photochemistry is probably associated
them.

Jagger: The thymines are different
course.

Rupert: Yes. That is true. But
poly deoxy-GC polymer seems to dev
the lesion upon irradiation (altho
weakly) even without any thymine be
present. I am afraid we still have no
consistent story regarding the precur
of the lesions. We can quote at least
experiment that will make difficulties
any theory we have thought of. Ma
some experiments are misleading.

Errera[9] : When you had short illu
nation times, there was a small induc
period. Do you know what that mea

Rupert: I think this just means
some kind of multiple damage exists,
that it takes time before fully repai
units appear. For one thing, the prese
of the lag depends on the UV dose gi
the DNA. For the streptomycin mar
reduced to 1% activity, the lag is alw
seen. If you slow down recovery by red
ing the concentration of enzymes, the
duction period is stretched out in the sa
proportion as the rest of the curve. H
ever, at a lower UV dose, correspondin
10% activity, no induction period can
seen regardless of enzyme concentrati
The activity goes up with constant sl
from the beginning. This is also fou
with other markers.

Another point. It doesn't seem to t
time for the enzyme to get going after
applying the light. If you stop the recov
by interrupting the light and then resu
illumination, recovery resumes at the sa
rate it left off. On the other hand, if
photoreactivate to maximum and then
more irradiated DNA, the recovery of

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[6] L. S. Kelly, University of California, Berk

[7] D. R. Krieg, Oak Ridge National Labora

[8] J. Jagger, Oak Ridge National Laboratory

[9] M. Errera, Université Libre de Bruxelles

ed material again shows a lag at the nning. The lag seems associated with first stages of recovery of the DNA. this is consistent with repair of mul- damage as the cause of the induction od.

ARMUR[10] : Are there any differences ie photoreactivation of various genetic kers present in the same irradiated ıophilus *influenzae* transforming DNA aration. In some preliminary experi- ts we have found that in the photo- tivation of some UV-inactivated *Bacil- subtilis* markers, the recoveries of the sforming activity were significantly erent.

UPERT: This should be given more y before I answer too definitely. We e worked mostly with the streptomycin ker, but we have spot-checked a num- of others. For markers having about same sensitivity, recovery is similar for same UV dose. Markers which are in- sitive, and require a heavy dose to bring n down to a certain activity level, seem how less recovery than more sensitive s inactivated the same amount. But don't have sufficient data to make a natic statement.

UCOFF[11] : In view of the possible utionary significance of this enzyme, onder whether a low dose of UV radia- would increase the enzymatic level in s?

UPERT: This is a good idea and ought e tried.

EINMETS: The system seems to be e complex than you can anticipate. did some photoreactivation experi- nts at low temperatures, which indi- ed that photoreactivating light at the n temperatures, had only little effect on bility of *E. coli*. However, in a frozen e, we found extensive inactivation.

LKIND[12] : You mentioned that the X-ray eriments have not yet been done. uld you speculate on the relationships veen the modes of action of X rays and in view of the fairly general observa- that it is not possible to photoreacti- X-ray killing?

UPERT: I should say this means that rradiation produces enough damage ch is different from the photoreactivable UV damage so that you cannot bring the system back to a viable state afterward. Mixed in with this irreversible damage there may also be some damage, due to excitations, which is the same as photoreactivable UV damage. This is the rationale behind attempting the experiments with Dr. Gray.

Marmur: I would assume that since the X-ray damage is more likely to cause backbone breakage of the DNA molecule, it is more unlikely to be reactivated by the photoreactivating enzyme than is UV damage which does not cause such damage at doses causing biological inactivation.

Gray[13] : If you deliberately break by sonication or some such method, does this interfere with that?

Rupert: No. The UV lesions that I am studying involve rather small segments of the molecule. As I said, if up to several per cent of the phosphate ester bonds are digested—which means breaking our molecule down into pieces averaging a few tens of nucleotides long—you haven't done anything to the competitive inhibitory power after irradiation.

Kelly: I asked my original question concerning X-irradiated DNA very specifically with regard to competitive inhibition and not restoration, because I wondered whether you might have a case similar to the bromouracil DNA where you do have the bonding and not the restoration.

Rupert: We will write you when we know.

Harm[14] : Is there any quantitative difference in the photoreactivable fraction of lethal damages, if you compare sunlight with UV of 254 mμ wave length?

Rupert: I am not sure. In a number of cases it has seemed that we got higher recovery from sunlight-inactivated than from 254 mμ-inactivated material. But one ought to study this specifically with artificial sources putting out 300 mμ radiation. You can't do a sunlight experiment anytime you wish, or even repeat one precisely.

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[11] H. S. Ducoff, University of Illinois.

[12] M. M. Elkind, National Cancer Institute.

[13] L. H. Gray, Mt. Vernon Hospital, Northwood.

[14] W. Harm, University of Köln.

KAPLAN: As has already been mentioned here, Dr. Sheldon Greer, working in Zamenhof's laboratory, was the first to note a striking enhancement of the UV radiosensitivity of *E. coli* accompanying incorporation of the thymine analog, 5-bromouracil. Subsequently, Dr. Szybalski, who was then at Rutgers, and is now at Wisconsin, and our group also began to investigate this effect. Szybalski has also worked with UV, but has studied mammalian cells *in vitro*. We have worked with *E. coli* as Greer did, using both UV and X rays.

We started by confirming Greer's work, using a thymine-deficient mutant strain of *E. coli*. We also showed the effect is not limited to thymineless strains. Strains B/r and B will incorporate bromouracil and become radiosensitive when grown in the presence of sulfanilamide, which blocks *de novo* purine and pyrimidine synthesis. Without sulfanilamide there was no radiosensitization, indicating that this effect is not a consequence of the presence of the analog in the incubation medium. The thymineless strain exhibits a multihit type of X-ray survival curve. The incorporation of bromouracil into DNA of log phase cultures increases the slope of the exponential part of the survival curve by a factor of 1.8 to 2 in various experiments. The extrapolation number also decreases and in some instances we actually obtain an extrapolation value of 1, so that the multihit curve was converted to a single hit curve.

Szybalski has suggested that the effect has a good deal to do with the halogen atom in the pyrimidine analog. But we became intrigued with the possibility that the incorporation of any analog into DNA could alter its molecular structure in such a manner as to produce a detectable increase in radiosensitivity. On searching the literature we found certain candidate compounds among the purine analogs. The most useful of these thus far is the analog thioguanine. This has been shown by LePage to enter both RNA and DNA of ascites tumor cells.

We have confirmed this incorporation into DNA using S^{35}-labeled thioguanine. Under these conditions it was possible with a purineless mutant of *E. coli* K-12 to s
a very striking increase in radiosensit
to X rays, with a slope ratio of abo
with respect to controls.

The effect of thioguanine on UV se
tivity is very strikingly similar to tha
bromouracil. Thus, the radiosensiti
effect can be seen with both purine
pyrimidine analogs, providing that
are in fact incorporated into DNA.

Moreover, on a molar incorpora
basis, thioguanine is probably a good
more potent as a radiosensitizer t
bromouracil.

We found that the incorporation
bromouracil is essentially linear with t
in strain B/r. At about 2 hours, w
approximately 10% of thymine has b
replaced by bromouracil, there is no
tectable radiosensitization. The first de
table effect occurs at about 20% subst
tion of thymine and the effect increase
a maximum at the end of log phase gro
(about 8 hours), at which point the
placement of thymine by bromouraci
about 40%.

In contrast, maximal radiosensitiza
with thioguanine occurs when there is
tween 0.6 and 1.0% substitution of t
guanine calculated on the basis of guar
substitution. We presume that the s
stitution is for guanine, but at this
tremely low level it is not really poss
to be sure.

Since one of these is a purine and
other is a pyrimidine, one would exp
that they might enter DNA more or
independently, and that if they both w
incorporated into the DNA of the sa
organism one would get a further incre
in radiosensitivity. This is in fact w
happens, except that they do not go in
dependently.

Thioguanine tends to suppress the
corporation of bromouracil and thus
enhanced effect of both agents togethe
not the additive sum of the two indepe
ent effects.

Szybalski has said that labeling of b
strands of DNA is necessary to obt
radiosensitization with bromouracil.
have labeled both strands of DNA w
bromouracil first, then diluted ten-
and reincubated in thymine in orde

ybrid DNA, one strand of which con- d bromouracil and the other thymine. legree of labeling can be checked with esium chloride gradient technique. see that with X rays essentially inter- ate levels of radiosensitization are ob- d when hybrid DNA is present in the So some of the radiosensitization is but an appreciable portion of it still ins.

have been able to demonstrate a degree of incorporation of another g, 2,6-diaminopurine, in unchanged into *E. coli* DNA. This seemed worth on the basis of Freese's work indi- that this analog is mutagenic. It corporated at a level of around 1% t produces a perceptible increase in sensitivity to X rays (about a 20% ase in slope).

wever, 2-aminopurine produces no , either in terms of incorporation or rms of radiosensitization; and the nidine analog 5-fluorouracil, which is porated into RNA but not into DNA, is in fact a blocking agent for DNA esis, has no effect on radiosensitivity r our conditions.

us it would appear with this limited of analogs that every one that actu- goes into DNA produces a detectable ase in radiosensitivity. The same ma- s under conditions of nonincorpora- have no effect, and other analogs h are not incorporated do not seem active.

is of some importance that Dr. Ken- Smith in our laboratory has studied radiation-induced degradation of the ral absorptions of these analogs in ion, using both UV and X rays. It ars that thioguanine is remarkably sensitive to UV than any of the ral bases, and, that on exposure to ys, it is comparable to bromouracil thymine, both of which are rather ly sensitive.

LKIND: It seemed to me that in your s, the effect of the analogs in general V survival was more in terms of de- sing the shoulder—that is, reducing hitness number or the extrapolation ber while in the case of X irradiation, was an effect on what could be called sensitivity; namely, changes in slope that effects were significant.

This certainly indicates that the analogs are having an effect on X-ray sensitivity, but I wonder whether this is really true to the same extent.

On the other hand, in mammalian cells which have bromodeoxyuridine incorporated in their DNA, the effect on UV response is great in terms of sensitivity and not so great by comparison for X rays.

KAPLAN: I think you are right, that the principle of UV effect in the coli is a shift in the width of the shoulder. There is evidence in some of our experiments where the bromouracil labeling is quite high, that there is an increase of the slope as well. But the X-ray effect differs from the UV response in this regard.

I believe there has been very little work published on the effects of X rays on the mammalian cell. But as for the UV aspects, I think the principal effects occurred when the cells were carried to really extremely high levels of bromouracil incorporation, up in the range of 70 or 80% substitution of thymine.

Under those circumstances one is working with very sick cells also.

WOLFF[15]: Are the thymine analogs mutagenic, too?

KAPLAN: I don't know.

ENGEL[16]: We have been doing some similar work with *E. coli* and getting results which look very much like what you are getting.

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ne-Controlled Reactivation of Ultraviolet- ctivated Bacteriophage

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veral experimental approaches have n that the deoxyribonucleic acid A) of a bacteriophage is virtually all ensitive material for inactivation by violet light (UV), X rays, or decay of rporated P^{32} atoms. Whereas the sen- ities of different phages to X rays and ecay are usually proportional to their contents, this rule does not hold true e case of UV (Stent, '58). The rea- might be that in many cases there are rimposed obligatory reactivation proc- s upon the intrinsic UV sensitivity of DNA. For example, the two-component of UV-survival curves of phages T1, or T7, as well as the unusually low UV itivities of many temperate phages are idered to be due to some reactivating raction between phage and bacterial ome (Garen and Zinder, '55; Tessman Ozaki, '57). Results reported here give ence for another kind of reactivation, ing to the difference in UV sensitivity veen the closely related phages T2 and For any given survival level, T4 needs t twice the UV dose as does T2, al- gh these two phages contain approxi- ely equal amounts of DNA and do not r noticeably in their sensitivities to ys or P^{32} decay.

Modification of UV-sensitivity by a reactivation process

y crossing T2 with T4, Luria ('49) nd the UV sensitivity to be a recombin- heritable character, and Streisinger), in a more detailed analysis, was to show that it is controlled by a sin- gene, which he called *u*, closely linked e *r 17* locus of T2. This gene controls the extent of photoreactivation (PhR) uch a manner that without any excep- the greater UV sensitivity (correspond- to the u^+ allele) is accompanied by a higher PhR, and the smaller UV sensitivity (corresponding to the *u* allele) is accompanied by a lower PhR[1].

The mechanism by which a single gene difference is able to control these great differences in lethality and reactivability is of interest not only with respect to problems of gene physiology, but also as a model case for UV photobiology. To explain the phenomenon, obviously two principal possibilities exist:

(A) The primary photochemical alterations in the DNA differ greatly (either qualitatively or quantitatively), depending on whether the *u* or u^+ allele is present within the genome of the irradiated phage.

(B) The primary alterations are identical in the two phage types; however, the *u* gene controls intracellular processes deciding whether or not a given primary alteration will result in lethality. Such hypothetical processes might either revert the primary lesions or change their physical-chemical character to become nonlethal for the individual, or they might alter the physiological conditions within the host cell so that the otherwise lethal primary damages become nonlethal.

To discriminate between these two principal possibilities, it was necessary to study the UV survival of u^+ phage when infecting cells that contained additionally an intact *u* gene. In the following experiments this condition was approximated by cells infected with heavily UV-irradiated

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[1] The designation u^+ for the allele present in T2, and *u* for the one present in T4, which was introduced by Streisinger ('56), might be somewhat misleading in the context of the following results. Usually, the genotypic state which allows the gene to perform its function is designed as $^+$ (= wild type). In the case of the gene for UV-sensitivity, however, u^+ is the allelic state in which the reactivating action of the *u* cistron cannot be carried out.

T4 (about 70 lethal hits) at an average multiplicity of infection of three. Despite the occurrence of multiplicity reactivation (Luria, '47) the survival of T4 was negligible at this high UV dose; whereas the results have shown that the sensitivity of the *u* gene itself is sufficiently small to make sure that many of the cells obtain at least one undamaged *u* gene.

Materials and methods used in the experiments are described in a previous paper (Harm, '59). The following set of experiments was carried out:

(I) UV survival of T2, singly infecting normal *Escherichia coli* B cells;

(II) UV survival of T2, infecting *E. coli* B cells that were simultaneously infected with heavily (5 minutes) UV irradiated T2 at a multiplicity of about three;

(III) UV survival of T2, infecting *E. coli* B cells that were simultaneously infected with heavily (5 minutes) UV irradiated T4 at a multiplicity of about three;

(IV) UV survival of T4, singly infecting normal *E. coli* B cells;

(V) UV survival of T4, infecting *E. coli* B cells that were simultaneously infected with heavily (5 minutes) UV irradiated T4 at a multiplicity of about three.

The results of these experiments are presented in figure 1. The numbering of the curves refers to the above experimental conditions. Although the survival of T2 or T4 UV irradiated for 5 minutes was negligible compared to the survival of the phage under investigation, it should be noted that there are certain differences between curves I and II, and between IV and V. They are obviously due to the occurrence of multiplicity reactivation between the phage whose survival was studied and the heavily irradiated phage. It has been calculated from curves II and V that every lethal hit of the slightly UV-irradiated phage has a probability of about 15% of being reactivated by the heavily UV-irradiated phages infecting the same cell.

The slope of the T2-survival curve under condition (III), however, approximates the one characteristic for T4. Since the irradiation itself was identical under conditions (I), (II), and (III), the reduced slope under condition (III) can only be a consequence of the different environment

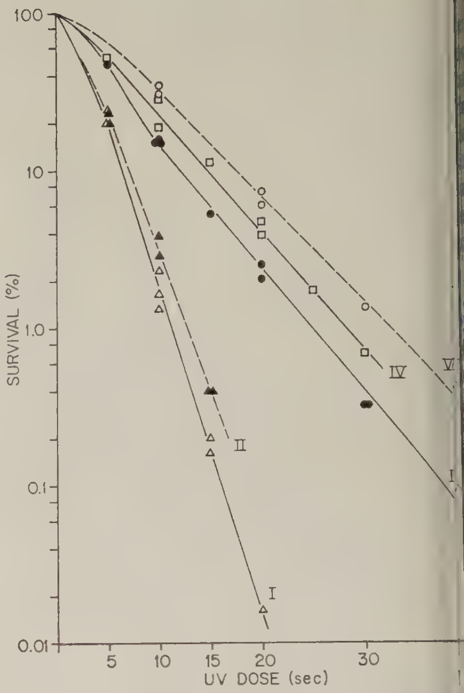


Fig. 1 UV survival of T2 and T4 undert ferent experimental conditions. The curves marked with numbers referring to the exr mental conditions described in the text.

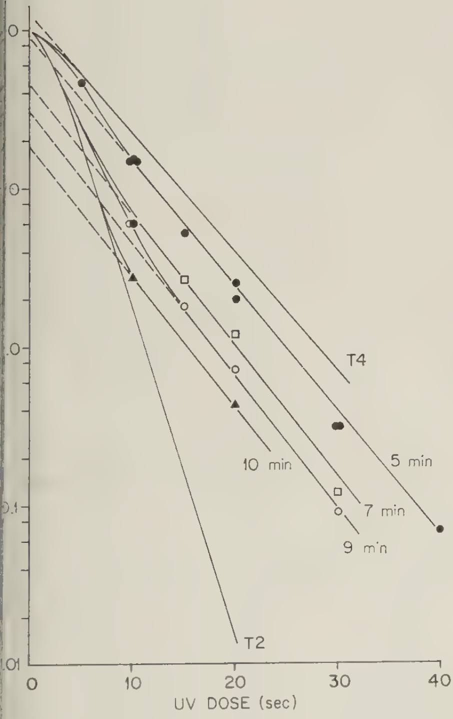
within the cell. This conclusion stron supports hypothesis (B), since it is s cient for the action of the *u* gene tha is present within the cell, but not ne sarily within the phage whose surviva being studied.

If this interpretation is correct, would expect that most of the phage ticles surviving according to curve III have the u^+ genotype, because the p ability for rescue of the *u* marker f the heavily irradiated phage by the viving phage would be small. To test theory, 10 plaques of about equal s originating from survivors of the hig UV dose under condition (III), were po in a buffer suspension and again UV ir iated. The survival was followed dow 10^{-3} and was found to be indistinguish from the original T2 survival. Evide none of the 10 phages from which plaques originated was genotypically otherwise the shape of the curve w have reflected some inhomogeneity of population with respect to UV sensiti

'e conclude, therefore, that an intact u
present within a cell is able to modify
survival of a u^+ phage infecting the
e complex by preventing lethality of
it half of the hits. For this effect we
l introduce the term "u-gene reactiva-
(uR), and we conclude that it oc-
obligatorily in T4, thus causing the
ced UV sensitivity of this phage as
pared with T2.

V sensitivity of the u gene function

ecause under experimental condition
) the u gene is introduced by the
vily UV-irradiated T4, the modified T2
ival should involve a certain probabil-
hat the u gene would be damaged.
would expect, therefore, that by fur-
increase of the T4-irradiation the frac-
of complexes able to perform uR will
ease. The experimental results, drawn
gure 2, are in accordance with this
ctation. The T4 UV-dose was raised



g. 2 UV survival of T2 under experimental
ition (III) with varied doses given to the
ily UV-irradiated T4. The 5-minute curve
entical with the one drawn in figure 1. The
hal survival curves for T2 and T4 are drawn
comparison.

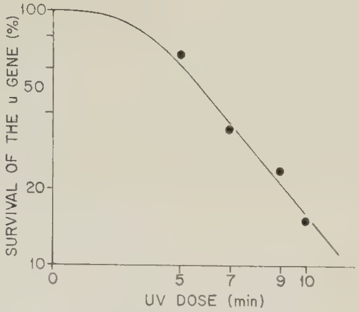


Fig. 3 UV survival of the phenotypic expression of the u gene as a function of dose. The points are the back extrapolation values of the curves in figure 2, relative to the back extrapolation value of the T4-curve.

from 5 minutes to 7, 9, and 10 minutes, but everything else was the same as under condition (III). It is obvious that the increase of T4 irradiation has no effect on the final slopes of the curves, but decreases the values extrapolated back to zero dose. These values are proportional to the fraction of complexes able to carry out uR.

These extrapolation values are a measure of the UV sensitivity of the u gene function. In figure 3, these values relative to the extrapolation value for the normal T4 curve are drawn as a function of dose. The shape of the curve, resembling a three-target survival, is about what one would expect for an average multiplicity of three, if the u gene function is inactivated with one-hit kinetics and if one undamaged u gene within the complex is sufficient for uR to take place. The slope of the linear part is $0.9 \pm 0.2\%$ of the slope of the T2 survival curve. This means that the sensitivity of the u-gene function is about 0.9% of the total phage sensitivity. It seems reasonable to compare the u-gene sensitivity with the T2 sensitivity rather than with the T4 sensitivity, since the curve for the u-gene survival itself cannot involve any uR. An objection might be that the slope of 0.9% does not express the true sensitivity of the u-gene function, because with high UV doses other lethal effects could occur, preventing DNA injection or other early steps of the infection process. In this case the 0.9% would be but a maximum estimation of the sensitivity of the u-gene function.

Relationship between uR and PhR

According to the results of Streisinger ('56) the great UV sensitivity (determined by u^+) is strongly correlated with a high extent of photoreactivability, and the small UV sensitivity (determined by u) with a low extent of photoreactivability. It is true, that under conditions of maximum PhR the T4 survival is still slightly higher than the T2 survival. However, it is reasonable to assume that the difference would completely disappear under conditions of saturated PhR. But these conditions cannot be realized experimentally, because of the superposition of an inactivating effect of visible light (Symonds and McCloy, '58).

If this assumption is correct, we may conclude: (1) All lethal damages reactivable by the u-gene action are also photoreactivable. (2) There is a fraction of damages, which are photoreactivable but not u-gene reactivable. This fraction is represented by the photoreactivability of T4, and amounts to about 10 to 15% of the primary lethal hits (i.e., lethal hits in T2). (3) The consequence of this relationship is that in case of obligatory uR (as in T4) the extent of PhR is small, and in the case of obligatory absence of uR (as in T2) the extent of PhR is high.

Experimental results with combined
and PhR, which need not be describe
detail, are in accordance with these
clusions. They have shown that the
survival under conditions of maxim
PhR is almost identical, whether or
the complexes were simultaneously
fected with heavily UV-irradiated T4.

Reactivability of the UV-inactivated rII-B function

All results described above concern
UV sensitivity of the phage as a re
ductive unit, i.e. an average for the t
sensitive material of the phage. The
lowing results, obtained by inactivatio
the *r*II-B cistron, indicate, however,
the extent of reactivability (PhR as wel
uR) may be different in different regi
of the phage genome.

The functions of the two *r*II-cistrons
known to be prerequisite for T4 to pro
gate within a cell of *E. coli* K12 (λ) (B
zer, '55, '57). The *r*II-mutants can in
these cells but usually cannot be re
cated. Krieg ('59) developed a method
which the UV survival of the function
these units can be measured when
irradiated wild-type phage infect cells
taining unirradiated *r*II-mutants.

TABLE 1

uR and PhR of UV hits preventing the rII-B function

Experiment number	Genotype		UV sensitivity of *r*II-B function relative to T2 sensitivity	
	UV-irradiated wild-type phage	Unirradiated *r*II-B mutant	Dark	Max. PhR
			%	%
477	u⁺	u⁺	2.4	2.2
480	u⁺	u⁺	2.8	2.0
481	u⁺	u⁺	3.2	2.2
483	u⁺	u⁺	2.9	2.2
487	u⁺	u⁺	2.5	2.2
488	u	u⁺	2.8	2.0
489	u	u⁺	2.6	2.0
502	u (T4)	u⁺	2.7	1.8
504	u (T4)	u⁺	2.8	1.9
Average:			2.75	2.05
471	u (T4)	u (T4 r102)	2.1	2.1
472	u (T4)	u (T4 r102)	2.1	2.1
499	u (T4)	u (T4 r102)	2.1	2.1
501	u⁺	u (T4 r102)	2.0	2.0
509	u⁺	u (T4 r102)	1.9	1.9
Average:			2.04	2.04

Reactivable sector: 0.7/2.75 = 0.25.

plex will liberate viable phage progeny as long as the rII-function of the wild- phage is not prevented by UV hits in this region.

or the following experiments the stock u^+ h^+ r^+, $\overline{\text{T2}}$ u^+ h r22, $\overline{\text{T2}}$ u h^+ r^+ of isinger ('56), T4 wild-type (Doermann) T4 r102 (Benzer, '55) were used. n the r22 and r102 markers are located he rII-B cistron. $\overline{\text{T2}}$ and T4 do not ex- le one another. The results concerning toreactivability and u-gene reactivabil- of the hits preventing the rII-B function, shown in table 1. They can be sum- rized as follows:

1) If both the irradiated wild-type ge and the unirradiated rII-mutant are u^+ genotype, the sensitivity of the rII-B ction is about 2.7 to 2.8% of the T2 sitivity.

2) The same result is obtained, if the diated wild-type phage is u, and the mutant u^+.

3) If the rII phage is genotypically u, matter whether the irradiated wild-type ge is u, or u^+, the sensitivity of the B function is only about 2.0 to 2.1 of T2 sensitivity.

4) Under conditions of maximum PhR sensitivity is about 2.0 to 2.1% for four combinations.

Ve conclude from these results that uR urs only if the u gene is in the unirradi- d phage, and that uR can reactivate ut 25% of the hits preventing the rII-B ction. PhR occurs to the same extent, only in those combinations where uR bsent. These experimental facts lead to the conclusion that 25% of the hits venting the rII-B function are reactiv- e by either uR or PhR, the other 75% not. Thus, the extent of reactivability considerably less than that found for average of lethal hits in the total phage ome.

The small amount of uR of the function- venting hits in rII-B is also the reason y uR cannot be observed when only the -irradiated phage was u. It can be cal- ated that there is hardly a difference in be to be expected, as the probability s that the u-gene function itself is dam- d.

Reactivability in the "vulnerable centers"

According to theoretical work of Barricelli ('56) the shape of multicomplex-survival curves of T2 (Dulbecco, '52) can be explained by the assumption of three so-called "vulnerable centers," which together make up about 20% of the total phage sensitivity. Experiments on multiplicity reactivation with T4 (Harm, '56; Epstein, '58) can be interpreted similarly assuming three vulnerable centers with around 40% of the total T4 sensitivity. The two results are compatible with each other if we assume that lethal hits in vulnerable centers are not reactivable by uR, but have the same absolute sensitivity. On the other hand, Dulbecco ('52) found the multicomplex survival of T2 greatly photoreactivable, which means that the PhR of the vulnerable centers is obviously high.

It can be estimated that the UV hits in vulnerable centers, which are photoreactivable but presumedly not u-reactivable are sufficient to account for the difference in photoreactivability and u gene reactivability inherent to the total phage, as mentioned earlier.

If this estimation is correct, the conclusion is suggested that generally the photoreactivable and u-reactivable damages are in fact identical, and the two types of reactivation have similar mechanisms. It is only necessary to assume that there is a special reason that uR cannot take place in vulnerable centers. The reason for the high contribution of the vulnerable centers to the total phage sensitivity is their presumed preduplication function, by which functional damages, which otherwise are non-lethal, are lethal in this case. We can, therefore, interpret the non-uR of hits in vulnerable centers by the *ad-hoc* assumption that these early functions have to be performed by the vulnerable centers first, to enable the u gene to operate.

Experiments by Stahl *et al.* ('61) also support the idea that u-gene reactivable and photoreactivable damages are identical. They investigated the UV survival of $\overline{\text{T2}}$ u and $\overline{\text{T2}}$ u^+ phages, of which the thymine was fully substituted by 5-bromouracil. It was found that the sensitivities of u and u^+ phage became identical and that no PhR occurred. The simplest explanation for this behavior is that both photoreactivable and *u*-gene reactivable UV damages occur in thymine.

OPEN DISCUSSION

J. Setlow[2] : You have postulated that the *u* gene possibly makes an enzyme inside the cell. Have you any information or way of finding out whether the presence of UV damage in DNA is necessary for this enzyme to be made inside the cell?

Harm: I cannot answer this question from the results presented here, because our experimental conditions involved necessarily a highly UV-damaged T4-DNA.

J. Setlow: Could you inactivate those phages by some other agent which does not involve the photoreactivating enzyme as we know it, so that the *u* gene enters the cell carried by a phage inactivated by an agent other than UV?

Harm: One could do this, but one must have an agent by which one is sure that it does not give multiplicity reactivation with UV-irradiated T2. And, second, it must be some agent by which you are sure that the genome goes into the bacteria. I think UV of wave length 254 mμ is a very exceptional agent in this respect. The work of Winkler and Johns at Cal Tech has shown that, even if one simply changes the wave length, one finds that at a given survival level the fraction of phages able to inject their DNA changes considerably.

Winkler[3] : We irradiated a mixture of T2 and T4 at different wave lengths (235, 265, 305 mμ) and determined the killing-curves for both phages. The ratio of the UV-resistance of T2 to T4 was about 1:2.4 at 235 mμ and 265 mμ. But by 305 mμ irradiation, we got a different ratio. It was about 1:3.4. That means that by 305 mμ irradiation more *u*-gene-reactivable damages are produced than by 235 mμ or 265 mμ.

G. Failla[4] : Is anything known about the spontaneous mutation rate of T2 and T4? How do they compare?

Harm: I don't think that anybody looked very carefully on this. Dr. Krieg, do you know if there are any quantitative measurements made to compare the spontaneous mutation rate of T2 and T4?

Krieg[5] : I do not know of any syst
atic, thorough comparison of spontane
mutation rates in T2 and T4, but I susp
that Dr. Benzer has some information
the subject. One pertinent investigat
of his (Benzer, '61) concerns a compari
of spontaneous forward mutation rates
two *r*II sites in T4 and T6. Since T6
twice the UV sensitivity of T4 and is
T2 in this respect, the comparison is
tween a phage strain having (T4) and
lacking (T6) the *u* gene. The two s
studied were "hot spots," i.e., those wh
r mutations most frequently occur in
In one case, the corresponding site in
had the same mutability as in T4 and
the other case its mutability was lower
a factor of four. He has, however, int
preted this difference in a way that d
not involve the *u* gene.

Billen[6] : Would the *u*-gene express
occur in the T2 system if you were to
chloramphenicol immediately after ph
infection, allow the antibiotic to rem
for 30–60 minutes and then wash it o

Harm: Yes, it certainly would. We
some of the uR-experiments with 10 to
minutes adsorption at a concentration
25 γ/ml chloramphenicol, however, I th
this does not mean very much. Since
has to dilute out the antibiotic after
while in order to measure the surviva
would suppose that the *u* gene control
processes take place thereafter.

Hutchinson[7] : I would like to ask
Rupert if there is a coli strain that d
not have this photoreactivating enzy
I am intrigued by the fact that the ph
reactivating enzyme is tied up by the
mouracil DNA and there is a lack of re
tivation with the bromouracil phage.
is possible that the *u* gene allows the p
toreactivating enzyme to act without lig

Rupert[8] : I think Dr. Jagger is the
to tell you about photoreactivation in
ganisms. I don't know myself of any

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[2] J. K. Setlow, Oak Ridge National Laborat

[3] U. Winkler, California Institute of T
nology.

[4] G. Failla, Argonne National Laboratory.

[5] D. R. Krieg, Oak Ridge National Laborat

[6] D. Billen, National Science Foundation.

[7] F. Hutchinson, Yale University.

[8] C. S. Rupert, The Johns Hopkins Universi

that is not photoreactivating. Do
John?

GER[9] : No.

PLAN[10] : Is it technically feasible to
e a u^+ mutant of T4? If so, would
ot expect that its UV sensitive curve
be precisely that of T2?

RM: Yes. Actually we are going to
is experiment when I am back in
any, and I have already thought about
echnical possibilities. It will not be
because we have no method to select
$^+$. Therefore, we will have to use a
g mutagen and then test suspensions
dividual plaques for UV survival.
vant to be sure that among several
reds of T4 surviving the mutagenic
nent, we will find some u^+ mutants,
n will facilitate our work with uR.[11]

EB[12] : Two questions. First, can you
out the possibility that the *u* gene is
ed at the vulnerable center or one of
ulnerable centers?

RM: I think this is ruled out from
act that the sensitivity of the *u* gene
ion itself is only about 1% of the
phage sensitivity, whereas the three
rable centers together have about
So from this comparison, it seems
probable that the *u* gene itself is not
nerable center, since the criterion for
nerable center is its high UV sen-
ty.

EB: Then does B/r have a *u* gene?
photoreactivable?

TKIN[13] : Yes, it is.

RM: I think B/r does not have a *u*
If it would have one, the survival of
nd T4 should be identical when infect-
3/r. I don't know whether anybody
this, but I would expect that the
tivity differences between T2 and T4
he same in B/r as in B. A long time
we tried several coli host strains to
are T2 and T4 survival, but we didn't
any strain where the ratio between
lope and T4 slope was significantly
ent from that obtained with coli B
e host.

AY[14] : Isn't the capacity of the bacil-
support T2 changed if you add the *u*
in the T4?

RM: At least not very much at these
UV doses. If you were using unirradiated or slightly UV-irradiated T4, the capacity for T2 would be low. T4 excludes T2 to some extent. If one takes an equal input of T2 and T4 for the same host cell, one finds among the progeny about 90% T4 and 10% T2 or something like that. But we did not have any difficulties in this respect with the heavily UV-irradiated T4. I don't remember exactly, but the loss of infective centers was probably not more than 20%. In any case this should not influence our curves since a certain loss of capacity would concern all experimental points to the same extent.

KAPLAN: In terms of Dr. Rupert's model yesterday, it would appear that the photoreactivating enzyme goes through two steps, the first one being a combination with damaged DNA, and the second, a light-catalyzed release of the repaired DNA.

In this instance it appears that the *u* gene functions in the absence of a need for light. Therefore the question arises whether the *u* gene functions in some way to release DNA from the same enzyme. That is, the simplest approach would be to assume that the photoreactivating enzyme is present in the *E. coli* anyway and that it operates in both cases to repair the DNA.

But an alternative release mechanism is supplied by the *u* gene and thus light is not required. In other words, the *u* gene might involve in some way another enzymatic system or some other catalytic function which would separate the repaired DNA from the same photoreactivating enzyme.

Could this not be tested in Dr. Rupert's system by making extracts of bacteria that have been infected with T4 and thus contain the *u* gene?

J. SETLOW: I might say this is exactly the experiment that I have been trying to do and I haven't had success yet. I have made an extract from T4 infected coli and have tried to reactivate UV damage to transforming principle in the dark with this extract.

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[9] J. Jagger, Oak Ridge National Laboratory.

[10] H. S. Kaplan, Stanford University.

[11] Meanwhile the experiment has been done successfully. Among 250 individual plaques Harm found one which was T4 u^+ as judged by UV-sensitivity, PhR, and host range.

[12] M. Lieb, University of Southern California.

[13] E. M. Witkin, State University of N. Y.

[14] L. H. Gray, Mt. Vernon Hospital, Northwood.

The results are muddy at the moment because of the bacterial nuclease.

KAPLAN: But that is not quite the experiment.

J. SETLOW: The use of the *u* gene system to repair transforming DNA might enable us to determine whether, as Dr. Kaplan suggests, the photoreactivating enzyme causes DNA repair in the dark, while the *u* gene supplies only the release mechanism. Fractionation of the coli extract could answer this question.

KAPLAN: I think you are making things difficult for yourself, however, because you are trying to get both the photoreactive enzyme and the *u* gene out of the same cells. Possibly it would be easier to start with a potent preparation of photoreactivating enzyme and use it in the dark with your extract of *u* gene-containing bacteria.

J. SETLOW: But the photoreactivating enzyme is always going to be there. How are you going to make an extract without it? It comes from coli.

KAPLAN: This may be, but the extract you prepare may be extremely weak in this regard. Possibly in order to demonstrate *u*-gene effect, you should start with a known potent preparation of photoreactivating enzyme and use it in the dark. If you could demonstrate release of repaired DNA, then the *u*-gene function would be related to the release.

J. SETLOW: Yes.

HOWARD-FLANDERS[16]: Would you show us the data from which you conclude that the *u* gene does not act on the "vulnerable centers."

HARM: If one compares the monocomplex survival curve with the multicomplex survival curve of T2, two facts are relevant: (1) The slope of the multicomplex curve is only about 20% that of the monocomplex curve, (2) the multicomplex curve has a hump in the low-dose range which is absent or very small with the monocomplex curve. The slopes of multicomplex curves are almost independent of the multiplicity of infection, but the
hump becomes the more expressed
higher the multiplicity. This is supp
to be due to the presence of three diffe
vulnerable centers (Barricelli, '56), w
behave like the units in the original
theory by Luria and Dulbecco ('49).
in order to survive, a multicomplex ha
contain at least one of each of the t
vulnerable centers in undamaged co
tion.

As a first approximation the multic
plex survival reflects solely the sensit
of the three vulnerable centers, whe
the sensitivity of the other material
be neglected. This means that in T2
sensitivity of the vulnerable center
about 20% of the total phage sensiti
In T4, the multicomplex curve has a
40% the slope of the monocomplex cu
i.e., the three vulnerable centers of T4
supposed to have about 40% of the
T4 sensitivity. Taking into account
the monocomplex sensitivity of T4 is
about half that of T2, the absolute sens
ity of the three vulnerable centers r
be about the same in T2 and T4.
is the reason to assume that the vulner
centers are not reactivable by the *u*-
action.

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e Analysis of Ultraviolet Lesions in Bacteriophage by Cross Reactivation

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ltraviolet radiation (UV) has long been
rded as a promising method whereby
might uncover some of the subtler
cts of bacteriophage multiplication.
l ('59) has recently reviewed the various
ways in which UV has been used in
endeavor, and he has summarized the
ent notions as to the mechanisms
ch may underlie the rather extensive
now available. The great majority of
rmation involves the ways in which
aged phage particles may be reacti-
d and is therefore appropriate to this
posium. It is the purpose of this paper
nquire in some detail into one of the
ems that employs UV radiation for the
lysis of bacteriophage genetics and
tiplication. This system is designed to
stigate "cross reactivation" (CR), and
present discussion will deal exclusively
the pertinent investigations made
bacteriophage T4.

ross reactivation has also been called
rker rescue," a term readily understood
n the description of such an experi-
nt. The procedure is to irradiate one
etically homogeneous phage stock and
repare a population of mixedly infected
teria, i.e., infected by a single irradi-
phage particle and phage particles
n an unirradiated stock (carrier phage).
genotypes of the carrier phage and
irradiated phage differ by one or more
tant markers, and the large majority
rogeny phage have genotypes derived
tly from the unirradiated parental
ge. The objective is to determine the
nt to which a genetic marker (or
rkers) from the irradiated phage is con-
uted to the progeny population. Thus
test is made whether the genetic infor-
tion in a particular marker of the ir-
iated phage is rescued from the killed
ticle as a result of the multiplication of the unirradiated phage. This test is an estimate of the probability of marker rescue taking place. In host cells where rescue does occur, it may also be of interest to know how many copies of the rescued marker(s) are found in the phage progeny.

Experiments of this type differ in at least one important respect from multiplicity reactivation (MR) experiments in which each of the phages infecting a bacterium has been irradiated. From analysis of the latter type of experiment, it has become clear that UV inactivates, on the one hand, targets that are large (vulnerable centers) and which must evidently carry out functions *prior* to replication and recombination of phage genomes. On the other hand, a second class of damages is also uncovered; and it appears to be composed of many smaller damages which, in sum, represent the remaining 60% of the total target. The conditions for MR (i.e., that a bacterium infected with UV-irradiated phages produce active virus) are that (1) from the total set of input particles, at least one complete set of undamaged vulnerable centers be present in the cell to perform the premultiplication functions for the complex, and that (2) small damages have not caused homologous lesions in each of the particles of the input. If multiplication can begin, genetic recombination is sufficiently efficient to reconstitute a whole undamaged genome in the majority of such cases. (For a more complete discussion and theoretical treatment see Barricelli, '60.)

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[1] Most of the experiments were carried out at the University of Rochester by Martha Chase Epstein and the author. They will be published in more detail elsewhere. The work was supported by research grants C-2306 and C-4437 from the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

Nature of UV lesions

The previous discussion already points up the need to begin any analysis of UV experiments by making a set of definitions describing the types of radiation damages to be distinguished. In this connection, an adaptation of the standard target theory will be used. UV irradiation renders phage particles incapable of plaque formation as an exponential function of dose. The slight shoulder generally, although not always, observed in plotting the logarithm of surviving fraction against dose will be ignored here. It will be convenient to express UV doses in terms of phage-lethal hits (PLH) which are given by the negative natural logarithm of the surviving fraction. This value represents the average number per particle of *total lethal damages* resulting from a given radiation exposure. The total is composed of several types of damages. The most obvious kind of damage one would expect to find is one that interferes with the ability of the virus particle to adsorb onto a sensitive cell, or once adsorbed, to inject its genome into the bacterium. Such damages will be called *injection damages*, and are equivalent to the "early-step" damages of Harm ('58). Considering the types of damages that might be anticipated after injection, Symonds and McCloy ('58) and Barricelli ('60) suggested a terminology which will be adopted here. These authors distinguish between different kinds of lesions by asking whether they interfere with the replication of the damaged site itself (genetic damages), and/or whether they interfere with a viral particle's ability to carry out some heterocatalytic function on behalf of all the phages infecting a particular bacterium (functional damage). Using these criteria, three kinds of lesions in addition to injection damages are conceivable, namely, exclusively *genetic lesions*, exclusively *functional lesions*, and finally lesions that produce both a functional and a genetic disability. The last type will be referred to as *mixed lesions*.

From MR experiments alone it is difficult to determine whether damages are genetic, functional, or mixed. In CR
periments, however, any functional d
ciency resulting from a lesion will au
matically be supplied by the unirradia
carrier phage put into the bacterium, a
it then becomes possible to analyze da
ages for their replicational, or gene
properties only. It is especially because
this simplification that CR experime
have here been selected for closer scruti

CR experiments have been carried
largely by two methods having sufficien
different implications to call for separ
treatment. The first technique, wh
makes use of single-burst technique a
is restricted to low doses (maximum
50 PLH) will be called the *single-bu*
method, and the second, which utili
the *r*II-K12(λ) system discovered by B
zer ('57), will be known as the *r*II meth

Single-burst experiments

CR experiments using the single-bu
method were first reported by Doerma
Chase, and Stahl ('55), who develope
technique of infecting strain B of *Esc*
richia coli with irradiated[2] wild-type at l
multiplicity (about 0.2 phage per ba
rium) and a carrier phage at a multipli
of two to three. The carrier phage is
tinguished from the wild-type by th
mutant loci (*m*, *r*, and *tu* in these exp
ments). After disposing of the unadsor
phages with anti-T4 serum, the culture
diluted sufficiently so that when samp
of the diluted material are distributed
many individual tubes, only about 2
of the tubes will receive a mixedly infec
cell. These single-burst aliquots are in
bated until the infected bacteria h
lysed, and are then plated. Any pla
showing one or more of the wild-t
markers (m^+, r^+, or tu^+) are scored
the frequency of each of the possi
genotypes.

From such an experiment, 4 clas
of bursts can result, namely, the c
with (1) all three, (2) with any two
the three, (3) with any one of the th
and (4) with none of the three wild-t

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[2] All radiations used were administered f
a 15-watt, low-pressure mercury vapor l
(germicidal lamp).

rkers represented. The last category
not, of course, be distinguished from
sts which never received a wild-type
ticle. Therefore all calculations of
rker rescue are based only on those
sses in which at least one wild-type
rker was scored in the burst. This
lysis also assures that any damages
t entirely blocked injection of the ir-
iated phage will be excluded from con-
eration. Since functional impairments
also eliminated in CR, only the genetic-
nage component of the UV lesions is
asured by single-burst experiments.

ingle-burst experiments involving three
nbinations of genetic markers were re-
ted by Doermann, Chase, and Stahl
5). In one set, all three markers were
ked, in another two markers were linked
l the third was unlinked, and in the
set of experiments, all three markers
e unlinked, or at least behaved as
ugh they segregated independently of
another. From the various experi-
nts, extending over a dose range from
b to 50 PLH, the following conclusions
ld be drawn:

. One marker of the irradiated parent
y be completely absent from the burst,
nough another is represented in a nor-
l or nearly normal number of the prog-
particles. This result suggests that
part of the genome can be damaged
hout appreciably affecting another part.
e marker that is absent from the prog-
will be referred to as "knocked out."

2. When markers are genetically un-
ked, they are knocked out independently
each other.

3. If two markers are linked (12 map
its in this case), they are knocked out
a correlated manner. More distantly
ked markers (20 map units in this ex-
ple) behave as though they are un-
ked in the sense that they are knocked
independently of each other.

4. If one marker of a linked pair is
ocked out, but the second is present in
burst, the latter tends to appear in a
aller number of phages than a third
linked marker contributed by the ir-
diated parent to that same burst.

The interpretation advanced to account
these results is that a certain fraction of the total lesions are located as discrete damages in the genetic structure, and that genetic recombination is the mechanism by which surviving markers are rescued from the radiation-damaged genome.

Experiments with the rII method

More recently Doermann and Chase ('61) developed another technique which permits experiments to be carried out at much higher doses of radiation. This method makes use of the fact that *r*II mutants of T4 cannot make plaques on K12(λ) while *r*II$^+$ phages are able to do so. In the *r*II method wild-type phage is irradiated with UV and mixed with strain B of *E. coli* in such a ratio that only one or two wild-type particles will be adsorbed per bacterium. In practice, multiplicities of 0.2 to 0.6 were used, correcting for doubly infected bacteria on the assumption of a Poisson distribution. Simultaneously the bacteria are infected with two or three particles of unirradiated carrier phage of some *r*II genotype. The infected bacteria, after removal of unadsorbed virus with anti-T4 serum, are plated on K12(λ) before they have had a chance to lyse. Any bacterium liberating wild-type phage makes a plaque on K12(λ) while others do not. In this way it is possible to measure the probability that an irradiated wild-type phage will succeed in transmitting to the progeny the *r*II$^+$ allele of the *r*II mutant gene in the carrier. Thus the chance of rescuing, as a function of UV dose, the *r*II$^+$ allele of any *r*II marker put into the carrier can be rather accurately determined. Since it is possible to plate as many as 10^6 *r*II-producing bacteria on one plate without interfering with the assay of the r^+ phage, a very low fraction of rescues can be detected and consequently large doses of UV (10^3 or more PLH) can be utilized.

Single rII$^+$ marker rescue experiments. An important advantage of irradiating the wild-type phage in such a system is that the same irradiated samples can be used with a variety of carrier phages. In that way CR of a number of *r*II$^+$ markers was measured by making use of different aliquots of the same irradiated phage suspensions. The points and the correspond-

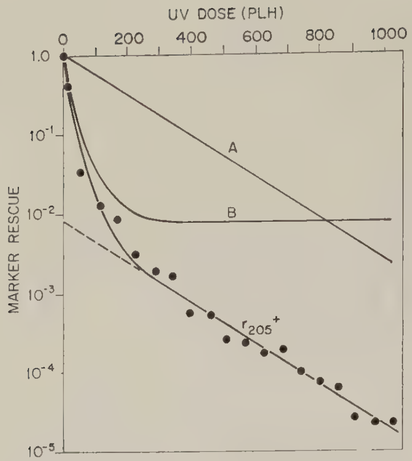

Fig. 1 Rescue of r_{205}^{+} measured by the *r*II method. The curve drawn through the points represents the probability of rescue of the r_{205}^{+} marker as a function of the UV dose. The curve labeled A represents the ultimate slope of the rescue curve, on the assumption that extrapolation (broken line) is justified. Curve B represents the survival curve divided by the A curve.

ing curve in figure 1 show the CR of the r_{205}^{+} locus of T4B.

For convenience in discussion, it will be useful to dissect the curve into two separate components. There appears to be an exponential portion which extrapolates back to a value of approximately 10^{-2} at zero dose. Assuming that extrapolation is justified, curve A has been drawn to represent one effect of UV radiation. Marker rescue values have been divided by corresponding values from curve A, leaving the second component of the marker rescue curve which is labeled B in the figure.

The single-burst data already suggested that UV causes discrete lesions in the genetic structure itself, and that rescue results from a crossover between the marker and the nearest hit to the left, and another crossover between the marker and the nearest hit to the right. In such a model only the hit nearest the marker on each side is of consequence, and for any additional radiation to reduce the probability of rescue, it would have to place another hit between the two hits surrounding the marker. Therefore with increasing doses of radiation, the sensitive target is expected to become progressively smaller as the hits are crowded nearer and nearer, and a curve which has an upward con-
cavity will result. The proposed inter
tation of curve B is that it represents
likelihood that a double crossover will
cur in such a way that it will rescue
undamaged marker from the surroun
hits.

Curve A, on the other hand, is in
preted as a measure of the lesions
render the marker unsusceptible to res
According to this interpretation, once
region beyond 300 PLH is reached w
the survival curve has assumed its
mate slope, the likelihood is negligible
any hit will be placed in a position
which it can reduce the probability of
cue without inactivating the marker it
In this range, practically all hits that
detectable in CR experiments inacti
the marker, or in some other way ren
it incapable of being rescued.

There are several ways in whic
marker could be completely excluded f
a chance of rescue.

1. Injection damages could conceiv
account for all or part of the slope of c
A. In single-burst experiments, injec
damages are not detected because the
are based only on yields containing at l
one marker from the irradiated ph
Such lesions would not, therefore, co
tute a component of the marker-res
curve when the single-burst method is
ployed. In contrast, injection dama
would constitute a component of
marker-rescue curve when the *r*II met
is used since the measure of marker
cue is based on the number of irradia
phage particles put into the experim
For example, if a UV lesion prevente
particle from adsorbing, the r^{+} marke
this particle would be included in the f
tion knocked out.

2. A second possibility to account
the slope of curve A is that it may, in wh
or in part, be due to the effective size of
lesion itself. This effective size might
due to the physical dimensions of the d
aged area, or alternatively it might re
sent a measure of some lower limit on
proximity of two rescuing exchanges.
ter additional data have been presen
discussion of the interpretation of b
curve A and curve B will be renewed.

Marker rescue curves of 5 other
markers have been determined and tre

TABLE 1

Characteristics of various single-rII+ rescue curves

Marker rescue curves have been drawn through points derived from CR experiments using different aliquots of the same irradiated wild-type phage for the corresponding points in the various curves. The best fit was made by eye. Each curve is based on values determined from 15–20 different doses of UV. The ultimate slopes (A curve, see fig. 1) were determined from data at 8–13 different doses which were higher than 300 PLH. All of the curves reached their ultimate slopes in the vicinity of 300 PLH.

Marker in carrier	Extrapolate of A curve	MLH/PLH[1]
r_{147}	0.7×10^{-2}	0.0062
r_{227}	1.0×10^{-2}	0.0060
r_{205}	0.8×10^{-2}	0.0059
r_{320}	0.6×10^{-2}	0.0059
r_{271}	0.9×10^{-2}	0.0055
r_{164}[2]	1.0×10^{-2}	0.0117

[1] Ratio of "marker-lethal hits" to phage-lethal hits, expressing the relative slopes of the A curve for a marker and the phage-survival curve.

[2] This marker is known to be a deletion in the A cistron of about 0.8 recombination units (Benzer, '57).

e the data in figure 1. The characteris- s of these curves are summarized in le 1. It is noted that the A curve com- nent of all 6 curves extrapolates to ap- oximately the same zero-dose intercept. rther, each marker rescue curve reaches ultimate slope at about the same UV se (300 PLH). Finally except for r_{164}^{+}, the markers are inactivated with out equal probability (0.0055 to 0.0062 LH per PLH, where MLH represents the s damaging the marker itself at any en UV dose, and is given by the nega- e natural logarithm of the surviving ction of markers which can be read di- tly from the A curve). The mutant r_{164} known to be a deletion covering about 3 recombination units (Benzer, '57), hereas all the others are presumed to be int mutations since they all have finite version rates. From these comparisons it ay be seen that except for r_{164}^{+} the curves e almost indistinguishable.

Multiple rII+ rescue experiments. Still ing the same samples of irradiated wild- pe phage, we determined marker rescue rves for a number of cases in which the multaneous rescue of more than one rII^{+} arker is involved. In this situation, both + markers must be rescued into the me phage particle in order to produce phage which can make a plaque on 2(λ). Representative rescue curves are awn in figure 2, and the characteristics 5 such curves are summarized in table 2.

Although it is for the present impossible to ascribe the A curve for single markers to genetic damages, the multiple-r^{+} curves do permit some estimate of genetic lesions. If we make the extreme assumption about the A curve, namely that all the hits inactivating single markers are due to injection damages, then the data in table 1 show that approximately 0.006 of all hits are in that category. But since the

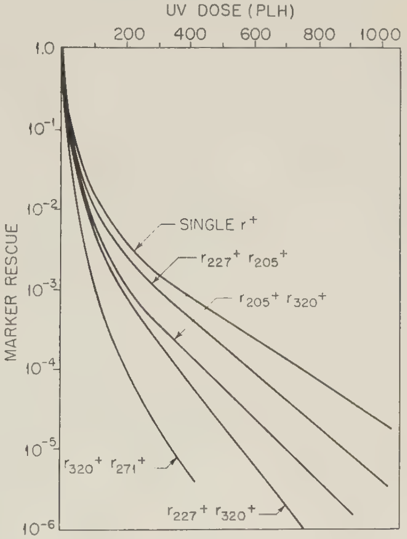

Fig. 2 Rescue of the average single rII^{+} marker and of representative multiple rII^{+} segments of the genome as a function of UV dose. The various markers used are indicated in the figure. For additional information consult table 2.

TABLE 2

Characteristics of various multiple-rII⁺ rescue curves

The data given are based on rescue curves as described in table 1. The same irradiated wild-type phage samples were used. In the multiple-rII$^+$ curves, however, the ultimate slopes were steeper, making it impossible to obtain reliable data at some of the high doses. The multiple-rII$^+$ rescue curves are thus based on 13 to 17 points each, and their ultimate slopes (A curves) are estimated from 7 to 11 points at doses higher than 300 PLH. Here, too, the ultimate slopes were invariably reached in the vicinity of 300 PLH.

Markers in carrier	Map interval[1]	Extrapolate of A curve	Total MLH/PLH[2]	Genetic MLH/PLH[3]	Hits per map unit
$r_{227}r_{205}$	0.26	1.0×10^{-2}	0.0078	0.0018	0.0069
$r_{205}r_{320}$	0.43	0.5×10^{-2}	0.0090	0.0030	0.0064
$r_{227}r_{320}$	0.75	0.8×10^{-2}	0.0120	0.0060	0.0080
$r_{227}r_{205}r_{320}$	0.75	0.5×10^{-2}	0.0120	0.0060	0.0080
$r_{147}r_{227}$	0.90	0.9×10^{-2}	0.0103	0.0043	0.0048

[1] From Chase and Doermann ('58).

[2] As in table 1.

[3] By subtracting 0.0060 from total MLH/PLH as discussed in text.

same irradiated wild-type phage samples were used in the multiple-rII$^+$ experiments given in figure 2 and table 2, the increased values of the ratio MLH/PLH must be due to the added sensitivity of the genetic material between the markers to be rescued. Similar to the single-rII$^+$ marker rescue curves, in all the double marker rescue curves (fig. 2) the ultimate slope is reached in the vicinity of 300 PLH. Furthermore, the A-curve extrapolations intercept the zero dose ordinate at approximately the same values as in the single rII$^+$ curves (between 0.5×10^{-2} and 1.0×10^{-2}). These similarities indicate that the B component of the survival curves is similar in all of the cases, and appear to give some justification to the dissection of these survival curves into an A and a B component. Since the curves differ only in ultimate slope (MLH/PLH), it seems reasonable to assume that in regions as short as those being considered here (less than one map unit), a hit between the two markers prevents rescuing both of them into the same phage particle. Such considerations appear to form a reasonable basis for calculating the fraction of the total damages per map unit and these estimates have been included in table 2. They show that for the rIIA cistron, one map unit will be hit with 0.0048 to 0.0080 of the total PLH.

One of the original objectives of the multiple-rII$^+$-rescue experiments was to obtain an estimate of the total map length of the genome. The slopes of the A curves for single markers suggested that very little the total damage could be accounted by injection damages (maximum of proximately 0.006). If it is assumed the purpose of calculation, first, that exclusively functional damages exist, a second, that hits are randomly distribu throughout the genetic map, the estim of the total map is simply the reciproc of the fraction of hits per map unit c culated in table 2. The values of the ciprocals are between 125 and 208 m units per total UV target.

This calculation is based on data tained using very short map intervals a are therefore almost completely correct for high negative interference (Chase a Doermann, '58). The total recombinati map of T4 when given in values correct for high negative interference (Barrice and Doermann, '60) must be appreciab longer than 200 units. One of the t assumptions therefore must be in err To ascribe to an appreciable fraction the damages an exclusively function character would only magnify the discre ancy between the already known m length and the CR estimate. Therefore t assumption of uniform sensitivity of t genome is probably unwarranted. Hen the rII region is probably more sensiti to UV than the average genome.

It should be recalled that the values 0.0048 to 0.0080 hits per map unit a based on the assumption that genetic h are point damages and that single mark knockout is due to injection damages.

extreme opposite is assumed, namely t no injection damages exist, but single-rker knockout is due to the effective ysical length of the lesions, a similar culation can be made. The result is ntical with the previous one, however; estimate of the genome is unrealis-ally short. It seems unavoidable that rII region of the recombination map is re sensitive to UV lesions than other ts of the genome. (See Barricelli and ermann, '61, for a more complete theo-ical treatment.)

Rescue of surrounded marker. While ne difficulties seem to arise when trying make precise quantitative determina-ns from the CR experiments, the results both the single-rII^+ and the multiple-$^+$ experiments give strong qualitative pport to the notion of rescue by double ssover. A third set of experiments uld also be mentioned in support of double-crossover hypothesis for marker cue. These experiments, like the first -CR experiments described, measure the bability of rescue of a single rII^+ marker. Here, however, another restriction is placed on the rescue. The irradiated phage is also mutant; one rII mutant is to the right and one is to the left of the rII^+ marker to be rescued. Thus r^+ phage is produced in a burst only when a double exchange succeeds in separating the centrally located r^+ marker from the surrounding rII loci. The latter act in one sense like genetic hits from which rescue must be accomplished. In figure 3, the data from one such experiment are shown. Here curve I represents the rescue of $r_{145}{}^+$ from wild-type phage as a function of dose. Curve II shows the rescue of the same locus from $r_{168}r_{145}{}^+r_{147}$. Because the surrounding markers are so closely linked to the centrally located r^+, that double crossovers are rare, wild-type phage are not produced in every mixedly infected bacterium. At zero UV-dose, the r^+ was separated at least once from the surrounding r's in only about 2.5% of the particles put into the cells. If the reducing target notion is correct, it would be expected that at some dose the surrounding hits on the genome would be closer to the $r_{145}{}^+$ than the surrounding r markers and the latter would no longer influence the probability of rescue. At that dose, the two survival curves I and II should become superimposable even though at zero dose they are far apart. If the curve for the surrounded case is divided by the curve for the single marker survival, the resulting curve should approach unity as the UV dose is increased. As seen in the figure, this expectation is borne out. In other words, at high doses, both of the two crossovers necessary to rescue the r^+ marker tend to occur within the interval between the surrounding r markers, producing an r^+ particle each time. Similar results were obtained using three other sets of markers. The dose at which the single r^+ and the surrounded r^+ rescues became equal was inversely related to the map interval between the markers. The major prediction of the double crossover hypothesis, that of reducing target size, was fulfilled, giving additional reason for its acceptance.

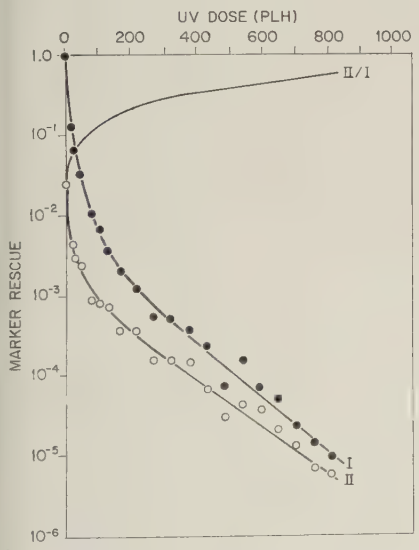

Fig. 3 Rescue of $r_{145}{}^+$ from irradiated wild-e and from irradiated $r_{168}r_{145}{}^+r_{147}$ as a function UV dose. Curve I represents the probability rescue from irradiated wild-type and curve II m the double-rII stock. The curve labeled II/I resents the influence of the two surrounding arkers on the likelihood of rescue as discussed text. It should be pointed out that r_{145} is dently a short deletion since it has no measur-le reversion rate (Benzer, '57).

Clone-size of rescued markers

The preceding experiments were concerned exclusively with measurements of

TABLE 3

Average clone size of markers in bursts where rescue occurs

Part A of the table is from single-burst CR experiments in which wild-type T4D was irradiated with UV and the frequencies of m_{42}^{+}, r_{48}^{+}, and tu_{42b}^{+} in the bursts were averaged. Part B is from *r*II-CR experiments in which wild-type T4D was irradiated with UV and single-bursts were plated on K12(λ) so that only the r_{47}^{+} allele would be scored. Except for the 48 PLH sample in part A, which contained only 65, each clone size determination is based on more than 100 clones.

A. Single-burst method		B. The *r*II method	
UV dose (PLH)	Clone size	UV dose (PLH)	Clone size
0	81	70	2.0
8	19	94	2.2
16	7.1	117	2.5
24	6.1	292	1.8
32	3.7	794	1.9
40	3.3		
48	1.7		
70	2.2		

the probability of rescue as a function of dose. In the hope of getting additional insight into the relationship of the rescued structure to the whole intracellular phage population, experiments were also done to find out how many copies of the rescued marker from one irradiated particle succeeded in getting into the mature infectious phages from one burst. At low doses, when the standard single-burst method is practicable, virtually any marker can be used. At higher doses, where rescues become rare events, a selective procedure is required. A simple modification of the *r*II method is efficient for this purpose. Instead of infected bacteria being plated before lysis, they are distributed into a large number of aliquots. The dilution made prior to distribution is such that an appreciable fraction of the aliquots contain no rescue clones. The aliquots are incubated until after lysis has occurred and then plated on K12(λ) a process which allows selective scoring of the *r*II⁺ particles in the progenies. Assuming a Poisson distribution, the number of clones can be calculated from the plates containing no plaques. The total number of plaques scored are then divided by the calculated number of clones to give the average clone size in bursts in which rescue occurred. This will be called the *r*II method for clone size determination.

Table 3 shows the results of experiments using the standard single-burst method for doses up to 70 PLH and the *r*II method for
considerably higher doses. It is noted th
the clone size drops off precipitously, ev
at low doses, so that at 48 PLH it has
ready been reduced to about two from
zero-dose value of 81. Thereafter the val
does not decrease farther. Even at
proximately 800 PLH the average clo
size observed was 1.9. This result is rath
surprising in view of Stahl's ('56) earl
observation that recombinants for two ve
closely linked markers in the absence
UV irradiation gave a clone size of or
1.2.

One plausible explanation of this
servation would depend on a difference
the average time during the latent peri
when rescue occurs as compared to t
average time when normal recombinati
takes place. For normal recombinants
appears as though most of the opportu
ties for exchange might occur late in t
growth cycle. This late production wou
result in many of the critical recombina
genomes being left in the vegetative po
at the time of lysis. For CR, on the co
trary, the average time of rescue ten
to be earlier and there is a greater like
hood that all copies would have been
moved from the pool and matured into
fectious phage particles. It is propos
that the reason for the later average
currence of the normal recombination
is that all prospective components of t
mating system become more numero
with time, and the opportunities for t
appropriate matings would then beco

TABLE 4

Clone size of r_{145}^+ rescued from irradiated wild-type and from irradiated $r_{168}r_{145}^+r_{147}$

The same stock of carrier phage T4Br_{145} was used in both experiments. In one, wild-type, nd in the other, $r_{168}r_{145}^+r_{147}$, was irradiated. The order of the markers is as given, with .14 map units between r_{168} and r_{145} and with 0.41 map units between r_{145} and r_{147}.[1] The umbers in parentheses indicate the number of clones on which the clone-size estimate is ased.

UV dose (PLH)	Clone size of r^+	
	UV-wild-type	UV-$r_{168}r_{145}^+r_{147}$
0	29 (88)	1.1 (51)
378	1.6 (160)	1.5 (94)
810	1.8 (230)[2]	1.5 (72)[2]

[1] From Chase and Doermann ('58).

[2] Two exceptionally large clones (277 and 124) were excluded from the calculation on he assumption that they represented two unirradiated wild-type contaminants introduced nto the adsorption mixture together with the carrier phage.

ter toward the end of the latent period. suggested that irradiated genomes however, unable to multiply, and efore the chance to rescue from the e UV-damaged genome is more uni- ly distributed throughout the latent d. In fact, it is known that the UV- tivated genomes are removed from vegetative pool and matured into in- ious (although incapable of independ- multiplication) phage particles (Her- and Burgi, '56; Tomizawa, '58). If UV-inactivated genome cannot repli- there would then appear to be a good ace that it would be swept out of the tative pool relatively early in the latent d with or without a rescue having n place.

ne test of this notion has been made. clone size of a surrounded rII$^+$ marker been measured as a function of dose in parison with the same marker rescued the wild-type phage. Table 4 shows data. When the wild-type was irradi- , the result was similar to the data n in table 3. The clone size at two doses was reduced to 1.6 and 1.8 from ro-dose value of 29. When the same ker was rescued from between two r rII markers, the clone size at zero was only 1.1, in agreement with l's earlier observation for a two-factor s. At both the higher doses, however, clone size was increased to 1.5, an in- se which is predicted by the hypothesis h invokes nonmultiplication of the diated particle to explain the clone-size .

DISCUSSION AND CONCLUSIONS

The principal conclusions from these data may now be summarized. From single-burst experiments it is clear that UV makes lesions in the genome and that markers are knocked out in a correlated way or independently of each other depending on the closeness of their genetic linkage. This result immediately suggests that the total UV target includes an appreciable fraction which suffers discrete genetic damages, and further that the rescue of genetic markers from such a damaged structure is brought about by genetic recombination which separates the markers from the surrounding damages.

High-dose experiments using the rII method support this hypothesis with three kinds of data:

1. In single rII$^+$ rescue, survival curves bend upward, reflecting reduction in size of the surviving target with progressively increasing doses. This curvature is predicted by the hypothesis which invokes double crossover as a rescuing mechanism. The rescue curves ultimately achieve a constant slope, suggesting a simple exponential function. This component (curve A, which is thought to represent the fraction of hits that inactivate the marker itself or its injection mechanism) may be removed from the over-all rescue curve by dividing that curve by the A curve. This procedure leaves curve B which is presumed to represent, as a function of dose, the likelihood of rescuing an unhit marker from the surrounding hits.

2. The second set of data in support of the double-crossover hypothesis for CR comes from a similar study of the simultaneous rescue of two closely linked $r\mathrm{II}^+$ markers. In those results, the A curves are found to be steeper than for single $r\mathrm{II}^+$ rescue, but the B curve components are superimposable with those for single markers. The steepness of the A curves is directly related to the genetic distance between the markers. This relationship re-emphasizes the notion that discrete genetic damages are under consideration. The similarity of the B curves in single- and multiple-$r\mathrm{II}^+$ rescue curves indicates that the rescue likelihood for unhit genetic intervals is the same in the two cases, as might be expected from the double-crossover hypothesis.

3. The third set of experiments that fit the hypothesis involve the rescue of a single $r\mathrm{II}^+$ marker from between two closely linked *r*II loci. Even at zero dose the probability of producing at least one wild-type particle in every mixed burst from such a cross may be considerably less than unity. In the example presented it is about 0.025. Thus at zero dose the rescue curve starts almost two orders of magnitude below the control in which the same marker is contributed by wild-type. The double-crossover hypothesis incorporates the idea of reducing target size, and predicts that when a dose is reached high enough to place hits as close to the r^+ allele as the surrounding *r*'s, the two rescue curves should become superimposable. In the example given such a dose is not reached, but the trend in the predicted direction is obvious.

When the data are considered from the quantitative point of view, the multiple $r\mathrm{II}^+$ rescue curves, on the basis of simple assumptions, give an estimate of the fraction of the total PLH which must hit one map unit of genome in the *r*II region. The estimates are from 0.0048 to 0.0080. Assuming that all hits are genetic and that the genome is uniformly sensitive, these estimates imply that the total length of the genome is between 208 and 125 map units. Since this is an unreasonably low estimate, the assumption of uniform sensitivity must be in error, and the *r*II region must be hypersensitive. (For more complete theoretical treatment see Barr
and Doermann, '61.)

One possible reason for the hypers
tivity of the *r*II region may be that i
it acts as a vulnerable center in multi
ity reactivation, as has already been
gested by Barricelli ('60). If that
true, then, as Harm ('61) has sugge
in this Symposium, the *r*II region w
have twice the usual sensitivity bec
damages in it would be immune to *u*-
reactivation whereas all those regions
in vulnerable centers would be subje
such reactivation in T4.

A final conclusion is that the UV-
aged genomes do not multiply, or, if
do, that it is only to a very limited ex
This is based on the observation that
at low doses the clone size of res
markers drops off precipitously, reac
a value of two already in the dose r
of 50 PLH. Also, the fact that the c
size of two appears to be the lower
regardless of dose (rather than 1.1 to
which is the clone size for rare reco
nants) is interpreted to mean that
damaged genome cannot multiply.
interpretation is borne out by the fur
observation that, in the surrounded ma
case, where the zero-dose clone size is
1.1, the high-dose rescue clone size
to 1.5.

OPEN DISCUSSION

J. SETLOW[3] : I would like to prese
shred of evidence that the model Dr. D
mann has presented for cross-reactiva
in phage can be used to explain the
inactivation of transformation princip

First of all, as Rupert and Goodgal
shown very clearly, when you plot
logarithm of surviving transforming a
ity versus UV dose, you get a curve
resembles the curves which Dr. Doerm
has shown for cross-reactivation exp
ments. The model that he has prese
here of continually shrinking target
is suggested by this logarithmic plot.
pert and Goodgal have shown that √N
versus UV dose on a linear plot is a stra
line.

Dr. Doermann's model has sugge
that the important thing for the prob

---

[3] J. K. Setlow, Oak Ridge National Labora

f incorporation of a marker is the dis-
e between the marker and the nearest
lesion. On this model, the target size
rinking as UV lesions are made in the
 molecule. After a given dose of UV,
rder to decrease further the probability
corporation of a marker such as strep-
ycin resistance, a lesion must occur
een the marker and the nearest lesion.
lesions outside this region do not
ge the probability of incorporation.
evidence suggesting that the cross-
tivation model may apply to TP con-
of the following:

hether the effective recombination dis-
e is terminated by a UV lesion or a
cation break should not matter with
ect to the resulting probability of in-
oration of the streptomycin marker.
he molecule is shortened the target for
is smaller, and we would expect to find
sonication of the molecule makes it
e difficult to inactivate by UV.

 addition to finding that sonication
eases the UV sensitivity, as I think
suggested by Dr. Marmur, I have been
to show that sonication and UV treat-
t are additive, in that if one sonicates
n to 10% survival and then applies
treatment, the survival curve behaves
hough one has used UV damage down
10% survival. Likewise, if one first
lies UV treatment and then follows
by sonication, the sonication survival
ve, which seems to obey the same dose
tion as UV, behaves as though the
A had been sonicated first down to
 survival.

ow, this would suggest that as far as
rporation of the streptomycin marker
oncerned, the probability of incorpora-
depends upon the distance between
marker and the nearest UV lesion or
of the molecule; and that the effect
the same whether the molecule is
pped or a UV lesion occurs in the same
e.

UPERT[4] : Several years ago Meselson
Stahl privately circulated a simple
ntitative theory which predicted the
endence of marker rescue on UV dose.
s theory followed Dr. Doermann's
del, considering that a mutant region
the genome (the "genetic marker") of
an inactivated phage could be "patched" into the genome of a viable phage by recombinational events — for simplicity, "breaks" — occurring at random with an average frequency B per unit length. In the irradiated genetic material, UV lesions ("hits") were pictured as also randomly sprinkled at an average frequency H per unit length, with rescue of the marker possible only when the nearest recombination "break" on both sides occurred nearer than the nearest UV "hit." The probability of rescue was calculated for the case where the marker was small compared with the distances between "hits" and between "breaks," and the total genome length was large compared with these distances, with its ends far away from the marker.

Starting at the mutation and going in one direction, the probability of encountering a recombination "break" before a UV "hit" is exactly like the probability of drawing a black ball from a mixture of black and white balls. It is $B/(B + H)$. Going in the opposite direction from the marker the probability is the same. The probability that "breaks" will be closer than "hits" on both sides (i.e., the probability of marker rescue) is the product of the two, which can be written $1/(1 + H/B)^2$. Assuming the number of "hits" per unit length is proportional to UV dose gives the same law Dr. Goodgal and I found experimentally for UV inactivation of transforming DNA; the activity of an irradiated genetic marker, expressed as a fraction of the unirradiated activity is $1/(1 + CD)^2$, where C is a constant for a particular genetic marker and D is the UV dose.

The Meselson-Stahl result can be extended a little if one considers that the nearest recombination "breaks" on each side of the marker delineate the target within which a UV "hit" must fall to be effective. The surviving fraction in such a collection of non-identical targets is

$$\int_{\lambda}^{L} w(l)\ e^{-Hl}dl,$$

with w(l) the distribution of target lengths, λ the shortest possible target and L the longest. The distribution of lengths produced by randomly breaking a number of lines, each of which contains a marked region, and then collecting the marked pieces is $w(l) = Kle^{-Bl}$, with the normalization constant K adjusted to make

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[4] C. S. Rupert, The Johns Hopkins University.

$$\int_{\lambda}^{L} w(l)\, dl = 1.$$

The case $\lambda = 0$, $L = \infty$ gives the Meselson-Stahl inverse square law. This is also obtained in any case as $H \rightarrow 0$ (i.e., at low UV doses) provided $\lambda \neq L$. If the marker covers an appreciable length so that $\lambda \neq 0$, with L remaining large, then at sufficiently high doses the integral changes from an inverse square to an approximately exponential function of H.

The inverse square law holds over several log units of inactivation of our *Hemophilus influenzae* markers. When you make the dose high enough, the theoretical curve fitting at low doses predicts higher survival than is found experimentally, but we do not reach an exponential region. However, others have found that for many pneumococcal markers (which require somewhat higher UV doses than the *Hemophilus*) the change from inverse square to exponential inactivation occurs rather early in the survival curve.

It should be clear that bacterial transformation is really nothing more than a genetic marker rescue—in this case rescue from a free DNA molecule (where the marker is entirely inactive) by recombination into a bacterial genome (where it will replicate and exert control over cellular processes). The transforming DNA is the analogue of an inactivated phage carrying some genetic marker, while the competent cell ready to be transformed is the analogue of a viable, unmarked phage inside an infected cell ready for recombination. The two systems should act in the same way in many respects. The similar UV survival curves and similar dependence of marker linkage on UV dose (the linkage studies in transformation of *H. influenzae* are being published by Dr. Goodgal) show that they do.

The question has of course come up whether the steadily decreasing UV sensitivity of transforming DNA as inactivation proceeds is primarily a fact of photochemistry or of biology. The answer here
is that at low doses we would expect
effect purely from biology.

One should try not to conclude too m
from such evidence. Dr. Marmur's res
seemed to indicate that at high doses
susceptibility to photochemistry also
creases with increasing dose. Perhaps
ing up enough damage on the DNA re
does affect the possibility of putting
further damage. But even without th
thanks to the clarifying concepts of
Doermann and his students—we sho
expect inactivation curves of the sh
observed.

Doermann: Would you think, th
that the reason for finding that there
different sensitivity for a streptomy
marker, for example, and ERY-1 or ER
is that the streptomycin marker is loca
in a part of the genome which tends t
broken into shorter molecules when
transforming principle is prepared?

Rupert: Yes. Certainly this very s
ple theory as it stands would predict
markers have the same sensitivity, wh
isn't so. In order to get different sensit
ties for two regions of the genome
would have to say that there are differ
ratios between the average number of
hits and the number of recombinat
"breaks" in these regions.

The genetic structure is not an am
phous line as pictured in these derivatio
The "hits" and "breaks," rather than
curring strictly at random, should dep
somewhat on the local structure. Just
the rII region acts differently from
average of the whole phage genome, dif
ent locations in the genome of the tra
forming system might have different c
acteristics in this respect.

J. Setlow: Alternatively there is
reason *a priori* to reject the idea t
breaks in the bacterial genome that
obtains when one makes a DNA prepa
tion are not random; so it is possible t
the pieces containing different markers
of different average lengths.

Rupert: Just one more bit of evide
that the decreasing UV sensitivity with
creasing dose isn't a matter of photoche
istry. Chemical inactivation of transfo
ing DNA by nitrous acid follows the sa
inverse square law as UV. I haven't ad

us acid to UV inactivation the way
Setlow has added UV and sonication,
he fact that the shapes of the dose-
t curves are the same suggests that
same underlying reason may be re-
sible—in which case it can't be photo-
ical.

ARCOVICH[5]: Could you evaluate the
ber of nucleotides which could be cal-
ted on the basis of these experiments
ctual size of the gene?

OERMANN: It would require a rather
number of rather dubious assump-
to make such a calculation from
e experiments. Since it would be neces-
to relate the value to total genetic
distance, all the assumptions implicit
enzer's fine structure analysis would
eeded. In addition the hypersensitiv-
o UV of the *r*II region would require
her parameter to relate the *r*II region
he rest of the genome. Finally the
tion of injection damages would have
e settled and an assumption would
to be made concerning the size of a
lesion. For these reasons I hesitate to
e such an estimate at this time.

OWARD-FLANDERS[6]: What is the fre-
cy with which hits that would inacti-
a section of chromosome would also
ce a switch near that point?

OERMANN: It seems clear that hits do
always induce a switch at the location
e hit, but it is impossible to say that
never do. To decide the frequency
which crossovers occur at the site
hit is too difficult for a decision at
ent.

he experiment which shows that UV
do not always cause a switch at the
tion of the hit was done by Dr. David
g ('59). He developed a system in
h he could measure rescue from an
cistron under conditions in which hits
e excluded from the cistron itself, but
located close to both of its ends. He
pared rescue of a single marker from
cistron with simultaneous rescue of
markers from within this undamaged
on. If UV always induces switches at
site of the hits, the rescue segment
ld extend from the hit located nearest
left end of the cistron to the hit located nearest the right end. This would include all the markers in the cistron so that single- and double-marker rescue ought to be equal. Krieg found double-marker rescue less frequently than single-marker rescue, however, indicating that the rescue switches do not always extend from hit to hit.

Isn't that the essence of your experiment, Dr. Krieg?

KRIEG[7]: Yes, it showed that you do not rescue the entire piece of genome between the two UV hits.

HOWARD-FLANDERS: It is sometimes assumed that multiplicity reactivation is almost 100% efficient for considerable sections of the genome. This would imply that lethal hits must frequently induce recombination so that they are not replicated.

DOERMANN: It is clear from various sources of data that progeny from multiplicity reactivation are highly recombinant. Barricelli ('60) has shown that the number of crossovers which have occurred are approximately the minimum which would be required to eliminate the UV lesions. This does not, however, prove that the recombinations are at the site of the hits themselves. From a theoretical treatment of crossreactivation, particularly rescue of surrounded markers, Barricelli and Doermann ('61) have reached the conclusion that the relative crossover frequency in the regions near the UV hits is significantly lower than in the central region of the undamaged segment between two neighboring hits.

KRIEG: My experiments that were just mentioned are not incompatible with the idea that UV may stimulate the recombination. It may indeed be, as several lines of evidence were mentioned, that the UV may stimulate recombination. My experiments merely established that the recombinations need not occur at the exact site of the UV hit. Perhaps it is possible that the UV hits may stimulate recombination within an interval near the hit.

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[5] Herbert Marcovich, Laboratoire Pasteur, Institut du Radium.

[6] P. Howard-Flanders, Yale University School of Medicine.

[7] D. R. Krieg, Oak Ridge National Laboratory.

HOWARD-FLANDERS: If you allow T2 complexes to develop for 7 minutes, then irradiate with UV doses of 10 or 15 phage lethal hits and then allow them to develop and lyse; the lysate will contain a very high proportion of viable phage. It is very difficult to detect phage with lethal lesions that are able to kill bacteria, but cannot form a plaque. The freedom from radiation injury in the progeny phage suggests that there must be some mechanism for the avoidance of the UV lesions during replication.

DOERMANN: What method did you use in looking at the progeny to detect the lesions?

HOWARD-FLANDERS: A host-killing assay. This is not very sensitive and would probably not detect 10% of injured phage.

DOERMANN: If we simply assume that the hits themselves don't replicate, then it is not unreasonable to think that there are dead particles there.

HOWARD-FLANDERS: Isn't that saying the same thing? If hits don't replicate and nearly all the progeny are viable the lesions must be avoided in replication.

DOERMANN: Yes, lesions are avoided in replication, but that the switches occur at the site of lesion is not necessarily implied.

MARMUR[8] : I wish to describe one experiment which may be pertinent. When we assay UV-treated transforming DNA with normal cells or with cells which themselves have been subjected to UV irradiation, the results are significantly different. Thus, UV-treated cells are less likely to be transformed by irradiated DNA than are normal cells. These results are consistent with the idea that the recipient cells may recover part of the damage to the irradiated transforming DNA, but are less likely to do so if it itself has suffered some UV damage.

However, we have been asked the question: What will happen to this curve if you UV-irradiate DNA and assay it on cells which have been UV treated? The curve went down this way.

In other words, the UV treatment to the cells is not increased, but decreases as the transforming DNA.

HOWARD-FLANDERS: It seems impo
at the present time to try to charact
lesions in the genome more closely. T
may, for example, prevent gene actio
may stop DNA replication, or may
other effects. Do you not feel that t
is evidence for avoidance of radiatio
jury in the T4 system?

DOERMANN: The question is sta
cally so difficult that I don't think
know.

HOWARD-FLANDERS: Hershey report
few years ago giving a dose equivale
50 phage-lethal UV hits to 5-minute
plexes. A proportion of the complexes
vived and after a considerable delay
an almost normal burst. At 5 min
there may be a number of replicas of
phage genome but even so, to reconst
the phage genome free from injury
then replicate it is a feat calling for a
effective mechanism for the avoidanc
the radiation injuries.

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tors Influencing the Survival of Bacteria after
osure to Ionizing Radiation

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is not possible for me to categorize
neatly summarize the responses of
ria to ionizing radiation. Bacteria
morphologically, biochemically, and
tically heterogeneous group that live
timate contact with the environment
are very responsive to changes in that
ronment. Over the years, depending
he bacterial strain chosen and the
ls of experimental technique, a be-
ering number of effects has been ob-
ed for bacteria exposed to ionizing
tion. Numerous reviews of this litera-
have been written, but these fre-
tly leave the reader with the opinion
can show anything you want with
eria." Such a statement is a tribute
to the diversity and adaptability of
erial forms and the diligence and per-
rance of radiation microbiologists. It
so a reflection of the fact that we are
ng way from a knowledge of the basic
erlying mechanisms governing the re-
se of these cells to radiation. It is
oubtedly true that, by choosing new
ins of bacteria and novel experimental
roaches, many more phenomena may
e described. Fortunately, many radia-
microbiologists feel that there are
ugh phenomena presently available and
only a detailed study of these will
d results that may lead us to the under-
g facts.

uch of the experimental work in radia-
bacteriology has been concentrated on
bacterium *Escherichia coli.* The choice
his organism has much in its favor.
easily grown and handled for radia-
experiments. Much is known about
iochemistry and, in recent years, its
etic constitution. However, its ex-
ely diverse metabolic capabilities and
eager nutritional requirements make

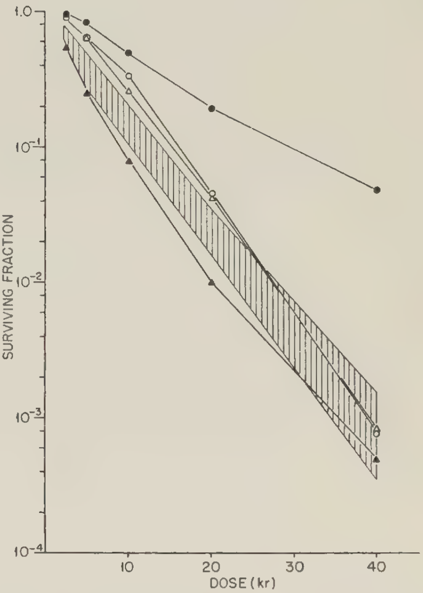

Fig. 1 Sensitivity of 4 "B/r" cultures to 250 kv X rays. Lined area represents range of results obtained with three "B" cultures. ●, B/r ORNL; ○, B/r Institut du Radium; △, B/r Cornell University; ▲, B/r CSH.

it unlike most cells of higher organisms, and it is therefore probably not the best organism for those who are anxious to extrapolate to higher cell forms, particularly to mammalian cells. Nevertheless it is a very useful organism and its study has given us many of the guiding ideas for future work. The study of this organism, particularly the strains B and B/r, in many laboratories throughout the world is an important part of radiation bacteriology. It is surprising then to find a great lack of agreement regarding the properties of these two strains. This stems, in part,

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[1] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

TABLE 1

Characteristics of B and B/r cultures[1]

Culture	"Snake" formation after 50 ergs of UV radiation	Final optical density[2]	Units of penicillin "G" required for 100% inhibition
B ORNL	+	0.309 ± 0.017	40
B CSH	+	0.303 ± 0.023	40
B Institut du Radium	+	0.297 ± 0.016	40
B/r ORNL	—	0.332 ± 0.034	60
B/r CSH	—	0.243 ± 0.035	120
B/r Institut du Radium	—	0.218 ± 0.030	120
B/r Cornell University	—	0.179 ± 0.025	60

[1] Taken from Adler and Haskins ('60).
[2] Based on 6 determinations per value. One S.E. is indicated.

from the fact that the cultures of *E. coli* B and B/r carried in different laboratories are indeed a phenotypically and genotypically heterogeneous group. This can be illustrated by comparing certain properties of these strains, properties that are important to radiation microbiologists, under standardized conditions (fig. 1, table 1). It can be seen that the various *E. coli* B/r cultures differ in their radiation sensitivity and the physiological characteristics examined (Adler and Haskins, '60).

Although the *E. coli* B cultures (fig. 1 and table 1) seem to be a relatively homogeneous group, there is a possibility that in different laboratories cultures labeled *E. coli* B are not the same since the dose required for UV inactivation of 99% of the cells has been reported as 4 ergs mm^{-2} (Matney *et al.*, '58) and also as 530 ergs mm^{-2} (Jagger, '60).

The current trend in radiation bacteriology is to relate sensitivity to the detailed metabolic and genetic mechanisms of cells and to generalize results from one laboratory to another. This can best be done when we are working with well-characterized cultures and are aware of the fact that similarly labeled cultures in other laboratories may be quite different. It would, perhaps, be wise to periodically recharacterize cultures and reserve the designations *E. coli* B and *E. coli* B/r for those bacteria having all of the characteristics described by Witkin ('47).

Another interesting example of the strain dependence of an important radiobiological phenomenon can be taken from unpublished data of Cromroy and Adler. In these experiments, 4 closely rela strains of *E. coli* were examined to est lish the magnitude of the oxygen eff Complete survival curves were run on organisms in the presence of oxygen a in nitrogen purified by pyrogallol wash and passage over hot copper turnings. T data are very briefly summarized in table It can be seen that the magnitude of oxygen effect is dependent on the part ular strain observed. Moreover, there is correlation between increasing radiati sensitivity and decreasing magnitude the effect. The strain *E. coli* B$_s$ isolated Hill ('58) from *E. coli* B is very sensit to X irradiation and shows an unusua small oxygen effect. We have tried modify the oxygen effect in the B$_s$ stra by alteration in the pre- and postirrad tion growth media. Although these man ulations do affect the absolute level survival, the dose reduction achieved removing oxygen remains unusually sma

These observations are another illust tion of the strain dependency of a m basic radiobiological phenomenon. It aga points out the need for caution in maki

TABLE 2

The oxygen effect in several E. coli strain

Organism	LD$_{37}$	DR aver
	kr	
E. coli B/r (ORNL)	13	3.
E. coli B (ORNL)	6	2.
E. coli B (Hill)	5	2
E. coli B$_s$ (Hill)	2.25	1.

Dose reduction factors and LD$_{37}$ are avera from at least two experiments for each organi alizations and the need for strict defi- is of the characteristics of the bac- n used for experiments.

ese strain differences among bacteria iot necessarily a cause for despair. are many examples in the radio- gical literature where a difference be- n two closely related strains, when rly understood, has been well ex- d. It can be expected that in the e, if time is taken to carefully define enotypic and phenotypic differences een strains, more basic knowledge will ined.

keeping with the philosophy that are too many unexplained phenom- n the literature of radiation micro- gy and that the introduction of a new s a grave responsibility, our group is ntly investigating some observations were first published several years ago. current work represents a cooperative rtaking of G. E. Stapleton, M. S. En- nd myself.

1951 Hollaender, Stapleton, and in ('51) observed that preirradiation th in the presence of glucose or under robic conditions made *E. coli* B/r cells tion-resistant as compared to cells n in nutrient broth. Birge and Tobias , were unable to get similar effects aploid yeast and suggested that the ts of Hollaender *et al.* might be ex- ed on the basis of multinuclearity or icellularity. Other investigators have rved similar effects in various strains *coli* (Schechtman, '60; Howard-Flan- and Alper, '57; Sargent, '61). The ts of anaerobiosis and growth in glu- are not completely independent phe- ena and the relation between them be developed further in this paper.

ne observation that *E. coli* cells grown ationary phase in a glucose-containing ient broth are resistant to ionizing ation in comparison with those grown utrient broth alone is an interesting nple of how a relatively small change nvironmental conditions can bring it a major change in radiation sensi- y. Growth of these cells in glucose- aining media cannot be considered an sual or unfavorable growth condition since the cells grow rapidly and to a high titer in such media.

Stapleton and Engel ('60) have been able to establish several factors that are involved in this phenomenon. By comparing cultures grown in peptone and peptone-glucose they have been able to show that there is a correlation between the pH to which the cells bring the medium and their radiation sensitivity. Cells that metabolize glucose and bring the pH of the medium to a low value become resistant. The nature of the phenomenon is illustrated in figures 2 and 3 taken from their work. It can be seen that there is an increase in multiplicity and a small decrease in slope for cells grown in glucose. The mechanism responsible for increased resistance to ionizing radiation also brings about an increase in resistance to ultraviolet light, heat, and hydrogen peroxide.

Chemical analysis for deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein, microscopic observations of living cells and cell wall-stained preparations indicate that the changes in resistance observed cannot be explained on the basis of gross structural abnormalities such as multicellular or multinuclear conditions or

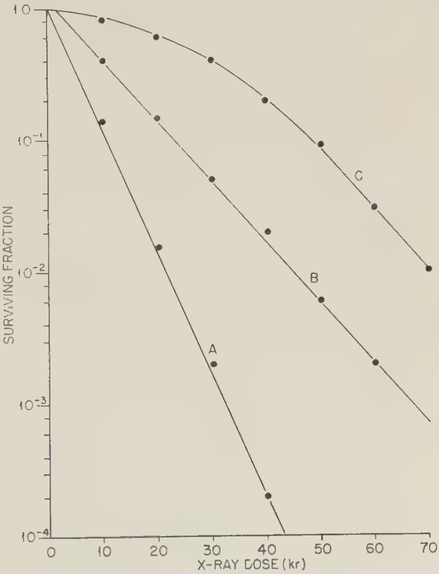


Fig. 2 Survival curves for *Escherichia coli* strain B/r (CSH) grown aerobically to pH 8.4 (A), pH 6.8 (B), and pH 4.8 (C). Initial pH of all media was 6.8.

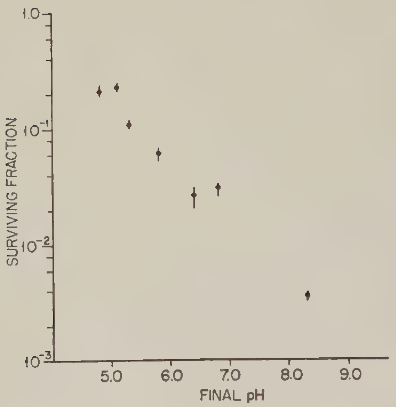


Fig. 3 Surviving fractions of *Escherichia coli* strain B/r (CSH) at a constant X-ray dose of 30 kr plotted as a function of the final pH of the growth medium. The mean of surviving fractions and their standard deviations are based on 5 or more experiments. The pH values were obtained empirically by altering the concentrations of the buffer and glucose in the growth medium.

on the basis of artifacts such as cell clumping.

The magnitude of the resistance phenomena can be altered by postirradiation plating conditions and is smaller for cells plated on synthetic minimal media (m-q) (fig. 4). Recently Engel established that the phenomenon can be observed for cells

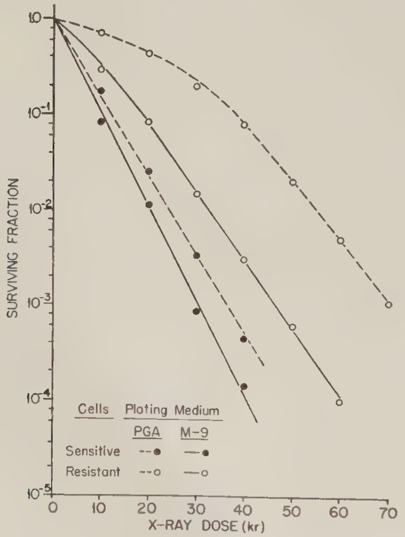


Fig. 4 X-ray sensitivity of *Escherichia coli* strain B/r (CSH) as a function of post-irradiation plating medium M-9, synthetic minimal medium; PGA, non-synthetic complete medium.

grown in completely synthetic media fore irradiation and that the presenc glucose is not a sufficient condition to duce resistant cells. The resistant sta only achieved if glucose is metabolize the presence of certain amino acids; thionine and glutamic or aspartic seem to be of particular importance.

Another approach to this problem been made through the use of the mu *E. coli* H_7. This mutant lacks the ab to synthesize porphyrin. It was isol and partially characterized by Belja ('55). It contains porphyrin enzy notably catalase and the cytochromes, if grown in the presence of exogene hemin.

Information on its radiation beha and physiology have been provided Adler ('58, '60) and Engel *et al.* (' Its important characteristics are sum rized in table 3. It is possible, by us this organism, to have the same gen material in a catalase-negative, chrome-negative protoplasm or a catal positive, cytochrome-positive one.

When H_7 cells are grown in the prese of peptone-glucose they do not become sistant unless hemin has also been pre (fig. 5). The failure of the cells grow glucose but in the absence of hemi become resistant cannot be attribute an inability to metabolize the sugar. Th cells do actively utilize the sugar lower the pH of the growth medium. parently, the presence of hemin is ne sary for the resistance phenomenon develop. Since the most striking effec hemin in these cells is to allow the de opment of catalase and cytochrome ac ity, it is implied that these enzyme syst are somehow involved in the resista mechanism. Further studies with H_7 al us to conclude that this is not necessa the case. Figure 6 shows that when

TABLE 3

Characteristics of Escherichia coli H_7[1]

Grown with hemin (H_7+)	Grown without hemin (H_7−)
Catalase positive	Catalase negative
Cytochromes positive	Cytochromes neg
Can respire	Cannot respire

[1] Taken from Adler ('60).

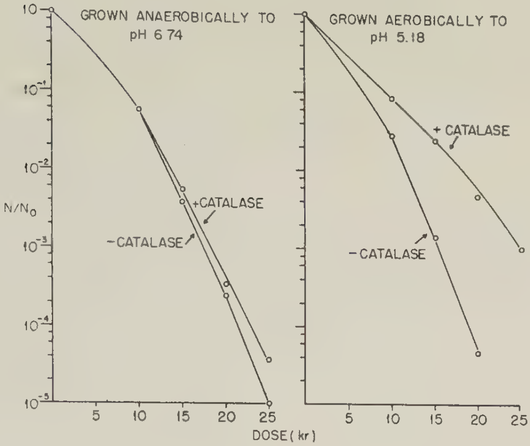
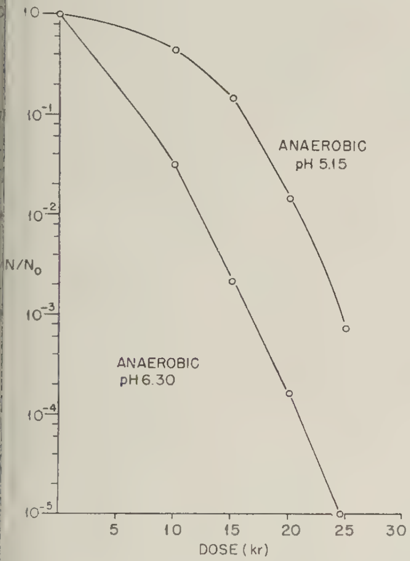


Fig. 5 The effect of growth in glucose to a low pH on the sensitivity of *E. coli* H_7 to X-irradiation.



g. 6 The development of resistance in an-
bically grown *E. coli* H_7 in the absence of
in.

diation growth is carried out under
erobic conditions, an effect of glucose
be observed even in the catalase-nega-
, cytochrome-negative cells. This effect
ot as large as that shown for hemin-
tive, aerobically grown cells but is
ımed to be a reflection of the same
c mechanism.

he observation that anaerobic growth
ned to enhance the glucose effect in
oli B/r was first made by Hollaender,
er, and Anderson ('51) and was considered by Sargent ('61) to be a necessary condition for showing this phenomenon. Experiments performed in our laboratory with cultures received from Sargent have shown that anaerobiosis is not an absolute requirement. However, the enhancing effect of anaerobiosis can be illustrated in the case of *E. coli* H_7 grown in hemin (fig. 7). It can be seen that, whereas cells grown to pH 6.8 aerobically are quite sensitive, cells grown to approximately the same pH anaerobically are resistant. They

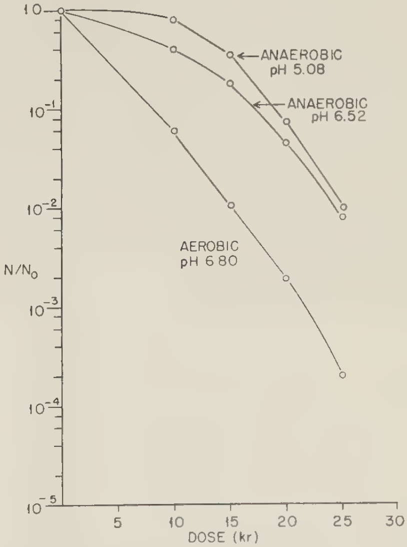


Fig. 7 The enhancement of the effect of glucose by anaerobiosis in *E. coli* H_7 grown with hemin.

are almost as resistant as if growth had occurred to a low pH (5.08).

To summarize these observations we may say that for cells grown aerobically, catalase and/or cytochrome activity is required for the glucose effect to be manifested. For cells grown anaerobically, these systems are not required. One possible explanation for this may be that the resistance mechanism does not develop and function well in the presence of hydrogen peroxide. Cells grown aerobically may produce hydrogen peroxide metabolically and thereby inhibit the development of the mechanism unless catalase is present to destroy the peroxide. Anaerobically, no hydrogen peroxide is produced and therefore there is no requirement for catalase. This hypothesis postulates a role for catalase during the preirradiation growth period. Catalase may, conceivably, be involved in determining radiation sensitivity in a more direct way by interacting with hydrogen peroxide produced by X irradiation in water.

It may be valuable to discuss at this point some recent data from our laboratory regarding the relation between catalase activity and sensitivity to ionizing radiation. Our interest in this subject stems from the observation made with catalase-negative *E. coli* H_7 cells that there is a post-irradiation inactivation which is caused by radiation-produced hydrogen peroxide and can be prevented by the addition of catalase (Adler, '58). Under the conditions of those experiments, catalase had no effect on the "prompt" inactivation caused by X irradiation.

Dr. Roderick Clayton and I have looked for a radiobiological role of catalase in the bacterium *Rhodopseudomonas spheroides*. By taking advantage of the work he done with this organism, particularly regard to the induced synthesis of catal it is possible to have bacterial cells which the catalase content varies fr 0.002% to 25% of the total protein (C ton, '60; Clayton and Smith, '60). have not been able to demonstrate t the level of catalase, between these tremes, has any influence on the radiat sensitivity of the organism. The most teresting aspect of this work has been finding that the ability to synthesize tein is inhibited immediately by X rays the same extent as the ability to fo colonies (Clayton and Adler, '61). Kin studies and studies of the effect of exter factors on the induced synthesis of ca lase after irradiation indicate that the pairment of protein synthesis may ar from damage in the genetic apparat It should be re-emphasized that th studies with *R. spheroides* are interest with respect to the mechanism of prot synthesis, and how radiation damages They do not indicate any radioprotect role for catalase present in the cell at time of irradiation.

Dr. Engel and I have done experime to evaluate the role of catalase in det mining radiation sensitivity in seve *E. coli* strains under a variety of conditio (Engel and Adler, '61). Tables 3 and show that there is no direct relation tween total catalase activity and resistan to ionizing radiation. They also show th there is no relation between catalase act ity and resistance to a challenge by hi levels of externally added peroxide. As matter of fact, cells grown in pepto glucose actually have a depressed catal activity but an enhanced resistance to

TABLE 4

Characteristics of peptone-grown E. coli strains[1]

Strain	Catalase activity[2]	Sensitivity to radiation[3]	Sensitivity[4] to H_2O_2
E. coli B_s (Hill)	184	4.55×10^{-6}	2
E. coli B (Hill)	173	8.35×10^{-3}	17
E. coli B/r (ORNL)	175	5.10×10^{-1}	45
E. coli H_7+	68	1.07×10^{-4}	—

[1] Table from Engel and Adler ('61).
[2] $\mu L\ O_2/min./10^{10}$ cells.
[3] Fraction surviving 20 kr (250 kvp, 30 mA, 3 mm Al).
[4] Percentage of population surviving 20-minute holding time in 0.6% H_2O_2 at 23° C.

TABLE 5

Characteristics of peptone-glucose grown E. coli strains[1]

Strain	Catalase activity[2]	Sensitivity to radiation[3]	Sensitivity[4] to H_2O_2
E. coli B_s (Hill)	24	7.70×10^{-5}	8
E. coli B (Hill)	33	2.40×10^{-2}	45
E. coli B/r (ORNL)	71	5.50×10^{-1}	70
E. coli H_7+	7.0	2.55×10^{-3}	—

[1] Table 2 from Engel and Adler, ('61).
[2] μL O_2/min./10^{10} cells.
[3] Fraction surviving 20 kr (250 kvp, 30 mA, 3 mm Al).
[4] Percentage of population surviving 20-minute holding time in 0.6% H_2O_2 at 23° C.

en peroxide! The data also show that e is a good correlation between sensi- y to ionizing radiation and hydrogen xide for the three closely related *E. coli* ns.

may be concluded then that the mere ence of catalase *per se,* does not auto- ically confer resistance to either ioniz- radiation or hydrogen peroxide. How- , the correlation between radiation stance and hydrogen peroxide resist- e does suggest that there may be some mon steps in inactivation brought t by these two reagents. It may even hat, under certain special conditions, ation-produced hydrogen peroxide may unt for some fraction of radiation age.

ne of the special conditions in which lase can have an appreciable protec- effect is illustrated in figure 8. It can seen that for catalase-negative *E. coli* H_7 cells grown anaerobically in a 0.1% glucose medium, the addition of catalase shortly before irradiation does not affect the prompt inactivation curve appreciably. However, figure 8b shows that, for cells grown in 1% glucose aerobically, added catalase has a marked protective effect. Apparently, only certain growth conditions allow the protective action of added catalase to be observed. Possibly, these growth conditions (1) change the permeability characteristics of the cell, allowing the catalase to reach critical sites or (2) provide an intracellular environment in which the catalase can be a particularly effective agent. It should be noted that the growth condition for which a protective action of catalase can be observed is one that leads to radiation resistance in naturally catalase-positive cells. It should also be pointed out that this effect of catalase is on the slope of the inactivation

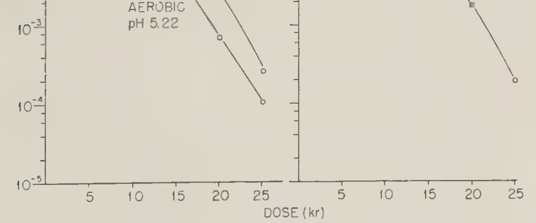


Fig. 8 The effect of catalase on the X-irradiation sensitivity of *E. coli* H_7 grown in the absence of hemin.

curve and this is not enough to account for the entire resistance phenomenon.

We can summarize our present understanding of the glucose-induced resistance phenomenon in the following way. An explanation on the basis of nuclear multiplicity such as proposed by Sargent ('61) for the very similar phenomenon that he studied, is not attractive to us. It is true that peptone-glucose grown cells contain approximately twice as much DNA per cell as peptone cells. However, one might expect some quantitative relation between this DNA increase and the extrapolation number observed for the inactivation curves. This is not the case for the data presented by Stapleton and Engel ('60) or in this paper (extrapolation number greater than 10).

The glucose-grown cells also contain more RNA and protein. In fact, they are of approximately twice the volume and therefore might be expected to contain, on a per cell basis, more of any major constituent measured. It is also true that these large cells appear to contain more internal granules when observed under phase optics. This results, in part at least, from the fact that they are larger cells and therefore more easily observed microscopically. Furthermore, the granules observed do not look like the structures observed by Mason and Powelson ('56). The structures observed by these authors are likely to be the true nuclear material of *E. coli* since they were observed to divide regularly in dividing cells. The granules observed by us may very well be storage material of the kind known to accumulate in bacterial cells grown in glucose-containing media (Knaysi, '51). Another factor arguing against the interpretation of this data on the basis of nuclear multiplicity is the observation that the magnitude of the resistance phenomenon and the extrapolation number is very much dependent on the postirradiation plating conditions (fig. 4).

It seems likely to us that the phenomenon described here can best be explained on the hypothesis that growth in glucose favors the synthesis of a protective system or a recovery system. On the basis of experimental evidence, we cannot make a clear choice between these two poss ties. The system may be a single c pound acting as a chemical protec agent or a more complex enzyme sys that allows the cell to repair or byp radiation-caused damage. In any case, synthesis of the system involves only erobic steps in the dissimilation of glu since it can develop in anaerobically gr cells. The work performed with grown in synthetic medium indicates particular amino acids are involved. experiment with the hemin-requiring tant of *E. coli* indicate that, if growth occurred under aerobic conditions, c lase and/or cytochromes may contri to the system. Catalase may be invol in the synthesis of the resistance sys by destroying metabolically produced drogen peroxide and also may someti be active as a protective agent during irradiation period. However, if growth occurred anaerobically, the resistance tem can develop in the absence of cata and cytochromes.

We hope that, by continuing this tailed study, we will one day be in a p tion to describe the mechanism of phenomenon in greater detail. It is v likely that it is not a generalization ap cable directly to other cell forms or e other genera of bacteria. However, wh understood to the best of our ability, may be able to predict in what cell ty it should work, in which it cannot wo and in which it may be made to work logical adjustment of experimental c ditions.

OPEN DISCUSSION

ALEXANDER[2]: We have done the op site to Dr. Adler. We have tried to se tize bacteria. We started this work on assumption that there were intracellu SH compounds that protect. We deci to remove them by pre-irradiation tre ments with iodoacetate. The treatme which we always gave before irradiat were such that they in no way decrea the viability of the organisms—in ot words, no killing, though I have no do that the metabolism of the cell was t porarily altered. The cells were usu ed in contact with the SH poison for minutes to 1 hour before irradiation then spun down and placed onto good n medium. We found that for *Pseudo-* *as fluorescens* which gives an expo- ial killing curve the slope was doubled retreatment with iodoacetamide (i.e., sensitivity was doubled). With micro- i both the shoulder and the linear part he dose-response curve were altered we obtained over-all sensitization to ays of as much as 7 times. This large factor for *Micrococcus sodonen-* is surprising since it contains only 0th of the number of SH groups than the *P. fluorescens* which is sensitized two times by SH poison.

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[2] Peter Alexander, Chester Beatty Research stitute, London.

OLLAENDER[3] : Do you ever leave the acetate there during the irradiation?

LEXANDER: We usually leave it there ause the cell returns to its normal sen- ity within 5 minutes or less after the acetate has been removed. So in most eriments we have it there during the diation, but it doesn't matter since we e done the necessary controls such as ing irradiated iodoacetate medium.

OLLAENDER: Very similar results can simply obtained with sodium bromide, we point out in our laboratory.

LEXANDER: An increased dose due to photoelectric absorption by iodine can explain these results since the total ount of iodoacetate bound in the cell ess than 0.1% of the dry weight.

RAY[4] : I should perhaps mention ethyl leimide which, as Dr. Bridges recently orted in Nature, sensitized *E. coli,* but material has to be given before ir- iation. I think we have to be a little tious perhaps when we consider these treatments. They may be influencing condition of the cell during the recov- period. To give the material after ir- liation might possibly be too late be- use it hasn't changed the metabolism in cell during the postirradiation period. Dr. Adler's summing up remarks in- ded the word "bypass." It may well be t some of the damage is in fact dimin- ed, I would think, by the development alternative pathways. Inasmuch as pro- n synthesis is usually required during t postirradiation period, it seems to me that it is somthing we should look at much more closely.

Several lines of recent work have shown that the ability of the cell to produce enzymes is also quite sensitive—in some cases I believe as sensitive as loss of colony-forming ability. This is shown by work published recently by Pauly on the production of adaptive enzymes. The sensitivity of this process was within a factor of 4 of the sensitivity for killing. The shoulder in the curves for resistant cells shown by Dr. Adler may reflect the need to inactivate various systems which are capable of producing adaptive enzymes and bypassing the radiation damage.

ALEXANDER: Experiments in our laboratory indicate that the sensitization by iodoacetate cannot be due to interference with repair processes that occur immediately after irradiation.

HEINMETS[5] : We tried to modify *E. coli* radiation sensitivity to UV radiation. One of the points of interest was the question how much did the cellular reserves have to do with this type of sensitivity. Endogenous respiration level was used as a measure. We tried to reduce it by continuous oxygenation. Even after 24 hours of aeration we were not able to change the radiation resistance. But extensive change was easily produced, either in photo-recovery or in metabolic-recovery. So indications are that this recovery effect is probably at least in that system not related to radiation resistance; however, repair process would depend very much on the level of the metabolic reserves.

ADLER: I perhaps should have indicated the nature of the changes in sensitivity to ultraviolet. *E. coli* B/r from Cold Spring Harbor does give a sigmoidal curve with UV dose. This is for cells grown in peptone.

Growth on glucose tends to extend the shoulder without any marked effect on the final slope.

For heat inactivation, *E. coli* B/r under our conditions, gives an exponential killing curve. After growth in glucose a shoulder-type curve is observed. The hydrogen-peroxide sensitivity curves are not precise enough to say much about this.

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[3] Alexander Hollaender, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

[4] L. H. Gray, Mt. Vernon Hospital, Northwood, England.

[5] F. Heinmets, Quartermaster Research and Engineering Center, Natick, Massachusetts.

von Borstel[6] : How does the rate of bacteria division vary with pH, that is with the two different growth conditions?

Adler: It is about identical. The resistance of the cells develops only after the cells have reached stationary phase. It develops actually several hours after the stationary phase has occurred and after the pH has dropped.

Dr. Engel did do experiments in trying to alter the sensitivity—by short-term incubations or treatments of either resistant or sensitive cells. Examples are those in which the cells that had become resistant were reincubated in various types of medium in an attempt to make them sensitive.

Our data at the moment look very much like this; although we can make the cells more sensitive, it seems as if in any of the conditions in which they become more sensitive there is also some cell division going on.

So we cannot say we have had success in sensitizing the existing cells except in the presence of cell division.

Engel[7] : Furthermore, the generation times of irradiated sensitive and resistant cells are the same. Therefore the difference in sensitivity cannot be related to differences in time available for repair.

Jagger[8] : You mentioned that in *Rhodopseudomonas* the protein synthesis sensitivity was comparable to the survival sensitivity. How does the survival of this organism compare with that of B and B/r?

Adler: It is similar to B. The LD_{50} for X rays was about 1600 r, making it quite sensitive. We can manipulate this also. For example, if we ask the cell to make protein immediately after it has been irradiated, it lacks this ability—or it has been impaired, depending on the dose. If, however, we put inducer in first and then irradiate, again the system is quite sensitive except if the process has gone on to the point where synthesis of protein has actually begun.

This is the time sequence. Initially there is very little catalase. At this point the hydrogen peroxide, which is the ind in this case, is added. There is a shor and then the synthesis of catalase be

If the radiation occurs any time be synthesis begins, this is a radiation-s tive process and the survival curve f is superimposable for at least the fir or 10 kr over that for colony forma But if the radiation occurs after synt begins there is a sudden step-wise ch in the direction of making the synt mechanism more radiation-resistant.

Kaplan[9] : Many years ago Stapleto his Ph.D. thesis described a remark change in the sensitivity of strain which occurs during growth, with sens ity during log phase and resistanc stationary phase.

There is a profound change in the sitivity of B/r, and this I am sure all microbiologically trained people in field have long known. We were cur as to why this should be, since in early phase, the organisms exhibit a degre radiosensitivity comparable to that strain B.

Originally there was the impression perhaps B/r contained more nucleic a as I believe Morse and Carter repo many years ago. Recent work of Gi and Alper, and others, as well as our investigations, indicates however that t is really no significant difference in D content between strain B and strain at any phase of the growth cycle.

Has Dr. Adler any observations with spect to hydrogen peroxide sensitivit B/r at different stages of the growth cy or similar observations relating to m bolic parameters which he was descri for this H_7 strain?

Adler: We of course took into con eration changes in sensitivity during growth phase and the one we are stud here is divorced from those.

Once his cultures reached statio phase in nutrient broth, the sensitivity

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[6] R. C. von Borstel, Oak Ridge National L ratory, Oak Ridge, Tennessee.

[7] M. S. Engel, Oak Ridge National Labora Oak Ridge, Tennessee.

[8] John Jagger, Oak Ridge National Labora Oak Ridge, Tennessee.

[9] Henry S. Kaplan, Stanford University, fornia.

ned constant. It is during this period
he stationary phase where, under the
ditions of growth in peptone and glu-
e, the sensitivity does become more
stant.

Ve have not determined the sensitivity
ıydrogen peroxide at different parts of
growth cycle. The only data that we
e on hydrogen peroxide sensitivity are
changes that I showed you depending
metabolic parameters in the *E. coli* B
B/r strains, and data on the H_7 strain,
icating that under certain growth con-
ons the sensitivity of the *E. coli* H_7
wn catalase-positive and negative is
ntical, both to hydrogen peroxide and
iation.

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X Rays Produce Chromosome Breakage in *cherichia coli* K-12?

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t is well established that the main ge-
ic effect of X rays is to induce chromo-
ne breakages which are followed by re-
r processes. In bacteria, the only genetic
ct of ionizing radiations observed so
consists of mutations induced in spe-
c loci; but changes in chromosome con-
ration, such as deletions or modifica-
ns in the sequence of the characters
e not yet been described. The exist-
e of sexuality in *Escherichia coli* K-12,
the facility with which it is possible
map the genetic determinants provide
with a good tool for such an investiga-
n.

This discussion concerns the analysis of
effects of X rays on the genetic ma-
al of *E. coli* K-12 HfrH. It is a part of
ore extensive work being published in
Annales de l'Institut Pasteur.

MATERIAL AND METHODS

he strains used are: (1) *E. coli* HfrH.
s strain is sensitive to streptomycin.
characters studied here are (a) Thr^+,
Leu^+, (c) Gal^+, (d) Try^+, and (e)
$^+$. They are transmitted at high fre-
ncy to the recombinants resulting from
nating with F− cells. They are listed
e according to their order of penetra-
into the female cell (see Wollman and
ob, '59). (a) Threonine; (b) leucine;
galactose; (d) tryptophan; (e) his-
ne.

2) *E. coli* PA 309 F−. This strain is
stant to streptomycin. It possesses sev-
auxotrophic characters, among which
those listed previously for prototrophy
he Hfr strain. Gal− is the inability to
ize galactose as a source of energy.

METHODS

he bacteria are allowed to grow in
h until a concentration of 2×10^8 cells/ml is obtained. Then the Hfr culture is irradiated with a Machlett machine (AEG 50–40 kv without additional filtration) and mixed with the F− culture in the proportion of one male to 10 females. The suspension is then mildly shaken at 37°C. for 90 minutes and plated on selective media. More technical details are given by Wollman and Jacob ('59). Media have been described in another paper (Marcovich, '60).

RESULTS

Dose-effect relationship in transmission of selected markers

HfrH bacteria are irradiated with increasing doses of X rays and diluted in a suspension of F− cells in the proportion of one HfrH for 10 F− cells. After 90 minutes at 37°C., during which the suspensions are gently agitated, they are spread on selective agar lacking the growth factor corresponding to the selected character. Streptomycin is added to kill the nonmated Hfr cells. For the killing effect irradiated Hfr bacteria are plated on broth agar. The results are the same if minimal synthetic medium is used.

The transmission of the selected markers is inhibited according to a single event dose-effect relationship. The radiosensitivity increases proportionally to the distance between the selected marker and the origin "O," which has been defined as the first portion of the Hfr chromosome to be injected into the female (Wollman and Jacob, '59) (fig. 1 and table 1). It had been shown previously that the inhibiting event is not linked to some damage of the genetic determinant itself, but that it is very

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[1] With the technical assistance of Melle R. Vayne, Service de Radiobiologie et de Cancérologie de l'Institut Pasteur, Paris.

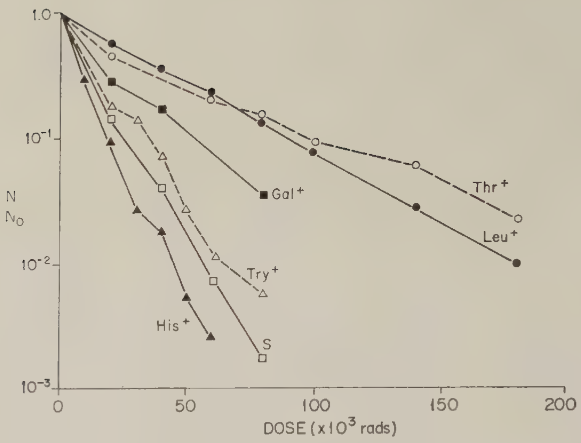


Fig. 1 Survival curves of *E. coli* K-12 HfrH irradiated bacteria and curves of decrease of the frequency of 5 selected markers after X irradiation.

likely to be related to some lesion of the transfer mechanism of the genetic material to the zygote (Marcovich, '60). This event is comparable to a chromosome break. After irradiation, all cells that have grown to form a colony on a given selective medium can be tested for the presence or absence of nonselected characters. The technique is to isolate the strain and test it on the appropriate medium. In figure 2 are plotted the frequencies of nonselected markers found, as a function of X-ray dose. Only the closed marker is shown because of the small numbers of colonies tested. It appears that the selected markers divide the chromosome into two parts. The "anterior" one between the origin "O" and the selected marker does not show any disturbance in the transmission of the nonselected markers and a "posterior" one, opposite to "O," in which the transmission of the nonselected markers may be disturbed. Moreover, the linkage of the Thr⁺, Leu⁺, and His⁺ markers—demonstrated by the number of colonies possessing them all at a frequency higher than if they were distributed at random—is not affected by irradiation. Figure 3 represents on a straight line the distances of the selected markers to the origin "O" of the chromosome in arbitrary units, proportional to the radiosensitivity of the transmission process. On the same diagram are plotted the distances of the closest nonselected markers to the selected ones estimated from data of the experiments shown figure 2. It may be concluded that radiosensitivity of the bacterial chro some as regards the transmission proc of the markers, selected or not, is ho geneous.

TABLE 1

Comparison between the radiosensitivity α[1] of transmission of selected markers from th HfrH cell to the female and their distance in minutes, to the origin "O" (see Wollman and Jacob, '59)

Markers	Thr⁺	Leu⁺	Gal⁺	Try⁺	H
α	22	25	48	67	1
Time in minutes	8	8.5	24	33	
α/Time	2.7	2.9	2	2.1	

[1] α is the inverse of the dose in rads w produces one inhibition event. The above va have been multiplied by 10^4.

Genetic analysis of Hfr cells surviving X irradiation

The foregoing results suggest that rays induce chromosome breaks in E. Hfr cells. If this were the case it mi be expected that translocations or d tions would occur and that the gen analysis of survivors of X-irradiated b teria would reveal them.

E. coli HfrH bacteria were irradia with a dose of X rays that left 10^{-5} s vivors. Twenty-seven colonies—the wh population of one petri dish to elimin

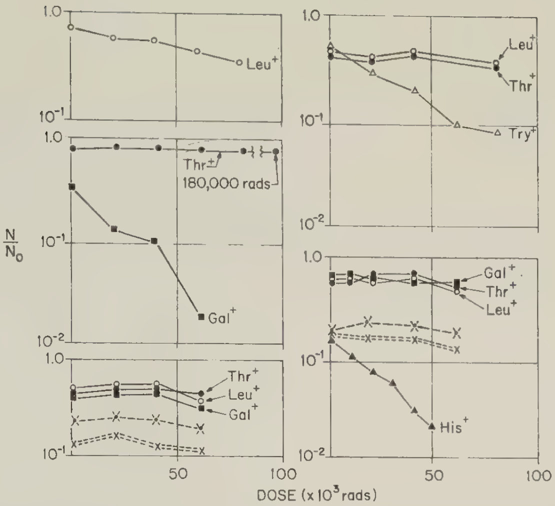
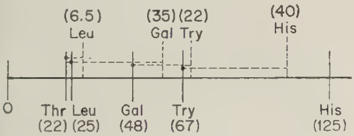


Fig. 2 Genetical analysis of the surviving recombinants of the experiments corresponding to figure 1. The colonies are isolated and, after subculturing, plated on selective media. Each compartment of this figure corresponds to the following selected markers: I, Thr$^+$; II, Leu$^+$; III, Gal$^+$; IV, Try$^+$; and V, His$^+$. The single dotted lines correspond to the colonies which actually possess the three nonselected markers, Thr$^+$, Leu$^+$, Gal$^+$. The double dotted lines correspond to the theoretical frequency assuming their random distribution.

need for selection—were then isolated l, after two passages in liquid broth e mated with the F $-$ strain. The re- ts are summarized in table 2.

wenty-two colonies behaved normally regards the frequency of recombinants sessing the different selected markers. e gave a much lower frequency of ombinants. However, the selected rkers demonstrated the existence of a gradient of frequency having the same direction as the gradient of the Hfr strain. The striking facts were the high frequency of the abnormal strains, characterized by the steepness of the gradient between the Thr$^+$, Leu$^+$ loci which are normally closely linked; at first sight these cell strains seem to be of the same type. Their isolation is too recent to have allowed much investigation to elucidate their nature. No translocations were noticed in the 27 strains examined in the "O"—His region of the chromosome.



ig. 3 Schematic representation of the dis- ces to the origin "O" of the selected markers, arbitrary units proportional to the slope of ir survival curves (lower numbers). Noted the values of the radiosensitivities of the selected markers deduced from their survival ves (fig. 2).

Action of glycerol

It is tempting to try to correlate the sensitivity of the selected markers to X rays to the actual amount of DNA of the chromosomes. However, an estimate of target size is very uncertain since radioprotectors can change the slopes of the inactivation curves. Such radioprotection

TABLE 2

Frequency of the selected markers Thr$^+$, Leu$^+$, Gal$^+$, Try$^+$, His$^+$ in the recombinations between the survivors of E. coli HfrH X-irradiated cells (10^{-5} survivors) and F$^-$ cells

	Thr$^+$	Leu$^+$	Gal$^+$	Try$^+$	His$^+$
Normal (mean of 22 crosses)	0.34	0.41	0.082	0.034	0.006
Abnormal (mean of 5 crosses)	0.027	0.0054	0.0013	0.0015	0.0009

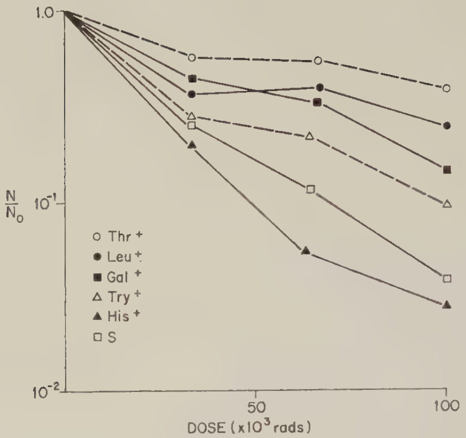


Fig. 4 HfrH cells irradiated in broth in the same conditions as in figure 1 but in the presence of 1 M glycerol.

may be observed if HfrH bacteria are irradiated with X rays in broth to which glycerol has been added at one molar concentration (fig. 4). This amount provides maximum protection to *E. coli* (Marcovich, '57). The dose reduction factor observed with this concentration of glycerol for both killing and recombination damage is the same, about 2.5.

DISCUSSION

1. The simplest hypothesis to explain the damage to the transmission of the markers to the recombinants is that X rays induce chromosome breaks.

On the basis of the results of figure 1, it appears that the radiosensitivity of one "breakage" event in the "O"— His region is about 3/2 of the one of one killing event. As this region is about one-half of the whole genetic map (see Wollman and Jacob, '59) and as the bacterial cell possesses two nuclear bodies in our conditions, one may estimate that there will occur 6 "breakage" events for each lethal one on the average per irradiated cell assuming that the sensitivity of the whole chromosome is homogeneous. Let us consider an irradiation that produces an average of about 12 lethal events; the survivors would be a little less than 10^{-5}.

According to the previous estimates, this figure would correspond to a mean number of 72 "breakage" events per cell or 36 per chromosome. If these eve are randomly distributed (and this is case for the "O" — His area) more t 95% of the cells would get between proximately 60 to 80 such events.

Let us consider one chromosome suppose that the reunion of the brok ends is operating at random. The pr ability of restoring the initial sequence genetic markers would be between 1/ to 1/40. The frequency of chromoson rearrangements would be very high. Th is not the case since no such modificati have been observed. The abnormal stra themselves seem to be concerned in mechanism of recombination and not the marker's sequence.

Four alternative explanations may count for damage to the transfer mec nism.

(a) X rays produce true breaks, the repairing process operates before fragments separate too far. There is so indication that the "breakage" events l for at least one hour. This observati results from a comparison between t radiosensitivities of the selected mark and their distance to the origin "O" c culated in minutes (table 1).

If a high-efficiency repairing process occur after X irradiation, the latest mark would be much less radiosensitive co pared to the earliest. This is obviously n the situation.

(b) The only viable sequence of mark for the Hfr cells is the one that is actua observed. Other strains have been fou to have another origin "O" or another rection of injection of the markers to t female, but their order is the same. have seen that even if this is the case random rejoining process of breaks wo restore the initial sequence with too l a probability compatible with the act sensitivity of the strain to inactivation.

(c) X rays produce some kind of inco plete breakage in the chromosome DN For instance, the lesion may consist o break of one of the two strands of bacterial DNA. The other strand might broken when the chromosome is injec from the Hfr cell to the female.

(d) X rays induce true breakages of bacterial DNA but the broken ends rem

e together, maintained by some non-
etic structure, a protein coat for ex-
ple.

xperiments with high LET particles
y give some information on hypotheses
nd d.

. Besides the damage to the process
transfer of the genetic markers of the
r cells to the recombinants, X rays have
n found to induce a mutation that is
ressed by a change of the frequency
h which the selected markers are trans-
ted. The gradient seems bigger than
h the normal strain. This effect is now
ng subjected to experimental analysis.

SUMMARY

Escherichia coli K-12 HfrH cells have
en irradiated with increasing doses of
rays. The frequency of the selected
arkers in the recombinants after mating
th an F − strain (PA 309) decreases ex-
nentially as a function of dose. The sen-
ivity of the process is proportional to the
tance of the locus of the marker from
e origin "O" of the chromosome. The
me finding is obtained when nonselected
arkers are concerned. In this case, the
tance to be considered is the one to the
arest selected marker. The radiosensi-
ity of the chromosome appears to be
mogeneous in the "O"—Histidine region.
Quantitative implications, based on the
sence of translocations in the recombi-
nts between F − cells and survivors of
adiated HfrH cells yield to the conclu-
n that X irradiation may produce points
frailty on the bacterial chromosome that
y result in true breaks during conju-
tion.

Aside from the damage to the transfer
chanism of the markers X rays seem
induce a genetic change that lowers the
obability for the markers to be injected
to the zygote.

OPEN DISCUSSION

AUERBACH[2]: I have never been con-
nced of the existence of rearrangements
bacteria; at least there was never any
oof.

Do you think this can have anything
do with the fact that bacterial chromo-
mes have no protein, and that the formation of rearrangements requires protein in the chromosome? This would support one of the interpretations for the necessity of protein synthesis for rearrangements?

MARCOVICH: I don't know. As a matter of fact, we have two explanations for this absence of translocation in this material. One is that perhaps the DNA of K-12 is two-stranded and that X ray would produce breakage on one of the strands. Then what we see is the recombination process which manifested itself when the chromosome is transferred to the zygote. This break is a point of frailty which may yield a complete breakage at the moment of transfer of the Hfr chromosome.

We are planning to use alpha rays which are more likely to induce breaks in the same allelic points of the DNA.

The other explanation should be that X rays induce true breaks, i.e. in both strands, but that there is a structure which holds the fragments together.

AUERBACH: What was the dose of X rays you gave for bacterial survival?

MARCOVICH: The dose was such that we had 10^{-5} survivals.

AUERBACH: That should break only one strand? It breaks whole thick chromosomes. If there can be no rearrangement in bacteria, isn't there then the possibility that this can explain the outstanding characteristic of bacterial chromosomes on which genes doing similar things are close together?

Couldn't this then have a historical reason, namely that these sequences arose by duplication and never had the chance to be scrambled up by rearrangements as they are in higher organisms?

MARCOVICH: Yes. A very striking fact is that the sequence is always the same, no matter how we select the Hfr strain. You may have changes in the direction in which the chromosome is injected or in the first factor to be injected. But the sequence is always the same.

One may raise the question of the observed sequence being the only viable one. If this was so, and if X radiation would have produced true breaks on the chromosomes, the hypothesis of rejoining at random would yield to a radiosensitivity of the lethal effect much higher than what is the case.

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[2] Charlotte Auerbach, Institute of Animal Genetics, Edinburgh.

Wolff[3] : The observation that you don't get nearly as many translocations as you would expect from the number of primary breaks is completely consistent with the information people working with chromosome aberrations have had for years.

It has been calculated that the numbers of breaks that go into the cell is far in excess of the numbers that finally appear as visible aberrations. To account for this we have had to postulate that the distance over which breaks can wander to find one another is a very important factor in these cases.

This distance factor, and the restitution of broken ends by which pieces go back together rather than recombine might also provide another explanation to the two which you have just given to Dr. Auerbach in regard to not getting translocations. That is, there is a linear chromosome that seems to be rather stretched out and not jumbled. It goes in in a linear fashion.

Therefore, the different broken ends won't be close to one another. A break can only be near its own end and restitution will be favored. There are similar indications of this—Tradescantia pollen grains. In Dr. Swanson's early work, while working with the sperm nucleus, where the chromosomes are relatively condensed, he found very few exchanges and translocations. The types of aberration he observed were for the most part simple breaks.

This may account for a lack of translocations and indicate that all that can occur is a restitution to original configuration.

Marmur[4] : In transforming experime one actually introduces into the recip cell what is in effect a portion of the D of the genome of the donor. The quest arises whether this DNA attaches itsel the genome of the recipient cell at so random spot or whether it is capable recognizing its corresponding niche. working with linked markers, it has b demonstrated that the transforming D goes to exactly the proper position in genome of the transformable cell.

Marcovich: When you say that thi DNA that knows the exact place wh it goes, is it a big piece or a small pie

In addition: Does it recognize its v in a single-stranded configuration or d ble-stranded?

Marmur: I don't think that this qu tion has yet been answered by anyone. have some very preliminary experime (in collaboration with Mr. C. Schildkr and Mr. J. Levine) indicating that DNA, when it enters transformable *Ba lus subtilis* cells, increases its buoyant d sity in a CsCl gradient. This is indicat that this DNA has suffered denaturati Whether it is this DNA that is potentia capable of carrying out the transformat is unknown.

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[3] S. Wolff, Oak Ridge National Laborator

[4] Julius Marmur, Brandeis University.

tirradiation Survival Kinetics of Mammalian ls Grown in Culture

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r some time, a favored conception has that a cell is rendered incapable of nited division by external X irradia- when its genetic apparatus suffers an opriate kind of unrepairable damage. proposal, which might be called the ome hypothesis," was adopted by and his associates to explain mam- an cell survival curves. Initially they osed a two-hit aberration model (Puck , '56) as an explanation of an appar- two-hit survival curve for HeLa S-3 but more recently (Puck, '60) have dened their model to include single-hit ts (e.g., point mutations), allelic in- ations (e.g., the recessive-dominant me for yeast proposed by Zirkle and as, '53), as well as nonspecific chromo- l damage.

a general way, the genome hypothesis several attributes. To begin with, it mechanistic appeal in that the integ- of the genetic apparatus of a cell must, ome degree, be essential to the life of the organism. It has been amply onstrated that radiation can give rise isualizable aberrations; there is good ence that mechanical failure of the tic apparatus can be one consequence -ray exposure; and in several systems, as been shown that ionizations pro- ed in or in close association with the eus are much more effective in disrupt- normal cell function than the same unt of energy absorbed by the cyto- m (von Borstel, '60). Another appeal is hypothesis is that cell death can be ed as the phenotypic expression of a ation event insofar as those lethal ts that do not produce visualizable mosomal defects are concerned. And ly, the genome hypothesis, as applied ammalian cells, contains an essential r in that it possesses the amplification properties required to explain how small amounts of absorbed energy can lead to profound effects. At least for cultured cells, mean lethal doses[1] (MLD's) are smaller by several orders of magnitude than those required to produce mean effects in intracellular macromolecules.

In spite of the foregoing, the genome hypothesis suffers from some inadequacies in connection with single-cell survival curves. For given cell lines, and even under what appear to be the same physiological and physical conditions, the extrapolation number, *n*, (Alper, Gillies and Elkind, '60) can vary by factors of 3 to 4 (R. Z. Lockart and M. M. Elkind, unpublished data). (The extrapolation number, with insufficient justification, is sometimes called the "hitness number.") MLD's on the other hand, vary by 50% or less. There doesn't seem to be any obvious relationship between ploidy or chromosome number and survival parameters. In addition, because the genome hypothesis as currently conceived is essentially a target hypothesis, it does not allow for significant interactions between DNA structures and components of either the surrounding nucleoplasm and/or cytoplasm. However, the potentiating effects of the halogenated analogues of thymidine (Djordjevic and Szybalski, '60; and W. Mohler and M. M. Elkind, unpublished data) implicate DNA as a molecule of primary concern. Several points are still not clear, however. We do not know, for example, if DNA-containing structures are primary targets, how they are organized, from a radiobiological point of view, to give rise to observed survival curves; whether or not the potentiating influence of these analogues results from their inhibition of restorative processes which occur at or shortly after the time of exposure; or whether or not these analogues produce "hot spots" of sensitivity at sites which would have otherwise made only a minor contribution to the total effective site structure of a cell.

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[1] In this paper, the term "mean lethal dose" (MLD) stands for the dose required to reduce the surviving fraction by a factor of 0.368 along the exponential portion of a survival curve. This definition has been customary and implies that the major mechanism by which cells are killed involves a set or sets of independent targets of equal sensitivity each of which can be inactivated by a single event. It is important to recognize that these assumptions may not be correct, in which event the MLD does not represent the dose required to score an average of one inactivating event per target.

In connection with this important question of where and what are the lethal sites in a cell, we undertook to study the kinetics of cells surviving an initial X-ray exposure. The rationale behind these experiments was the following. Almost all mammalian cell survival curves, determined *in vitro* or *in vivo,* have initially increasing negative slopes when plotted on semilogarithmic coordinates. That is, they are sigmoid in shape on linear coordinates. Whether or not the inactivation scheme is known, curves of this type indicate, at least, that damage must be accumulated before a lethal effect is produced. If the damage is assumed to be registered in the genetic apparatus, then surviving cells could be thought of as containing recessive damage. The purpose of our experiments was to see if sublethal damage was heritable as indicated by the survival properties of the progeny of surviving cells. What we found and previously described (Elkind and Sutton, '60) was that by about the time radiation-induced division delay appeared to be terminated the survival properties of surviving cells had returned to essentially those which characterized the initial population. Therefore, we concluded that surviving cells repaired their damage rapidly and that by the time a cell divided once after an initial exposure, it had eliminated its sublethal or recessive lethal damage.

The experiments to be described in this report are an extension of our original findings. They lead to some interesting interpretations with respect to the sensitive site structure in the progeny of surviving cells, and, by implication, in initially unirradiated cells.

MATERIALS AND METHODS

Our materials and methods have previously described (Elkind and Su '60). In brief, these consist of the monly employed bacteriological techni as modified for mammalian cells that attached to glass. The data to be descr were obtained with two cell lines der from the Chinese hamster. Line *A* o from ovarian tissue and line *V79-1* female lung tissue. Both lines appear f blastlike when spread on a glass sur and give rise to tight, discrete colo Because we use soft X rays (55 kv, 722 per minute), our usual procedure has to suck off the medium before the cells exposed. Since our initial report (El and Sutton, '60), we have found that in suspension reattach to glass surf within one hour at 37°C. During thi terval, cells growing in log phase w have increased in number by about Following exposure, fresh medium wa turned to the dishes.

One new aspect of our techniques sh be mentioned. For the separation of comprising a developing clone, as we for the preparation of stock-cell sus sions, the trypsinization procedure p ously described was used. As a result o post-trypsinization dilutions which found to be convenient, the final gro medium contained close to 50 µg per m 1–300 trypsin (Nutritional Biochemi Corp., U.S.A.). In order to eliminate differential effects resulting from try in the medium in only certain parts o experiment, all of the medium used in other parts of an experiment was sup mented with trypsin to the same ext The presence of trypsin had small eff on survival parameters. However, it nificantly improved the nutritional ca ity of our medium; an observation sistent with the finding that the gro factor contributed by dialyzed serum be replaced by the dialyzable product the proteolysis of serum proteins (Ea '60).

RESULTS

Survival independence

Much of the data we will describe cerns an analysis of the survival prope of populations of groups of cells. criterion of survival is the formation

e colony. Since one cell or a micro-
of viable cells would be expected to
into only one countable colony, the
sis of the survival curve obtained
a population of microclones, instead
ngle cells, depends on the survival
rties of the individual cells in the
ps of cells. In essence, therefore, the
sis of the postirradiation survival
ics of surviving cells and their prog-
onsists, in large part, of deducing rea-
ble estimations of the properties of
e cells from the survival properties of
lations of groups of cells.

referring to the experiments to be
ribed, we will frequently use the fol-
ng shorthand designations. In each
nce, after the final operation, the cells
ncubated at 37°C. for visible colony
ation.

Gr.—T ≡ A growth curve of unirradiated cells determined by incubating the cells at 37°C. and, at various times, respreading the cells by trypsinization.

Gr.—X ≡ Growth at 37°C. as a function of time followed by a fixed dose.

r.—Surv. ≡ Gr.—X except that graded doses are given to determine the survival curve.

Gr.—T, X ≡ Gr.—X except that cells are trypsinized before receiving a fixed dose.

—T, Surv. ≡ Gr.—Surv. except that cells are trypsinized before the survival curve exposures.

X—T ≡ A single fixed dose followed by varying periods of time at 37°C. after which the cells in developing clones are respread by trypsinization. This constitutes a growth curve for surviving cells.

X—X ≡ Two fixed doses separated by varying periods of time at 37°C.

X—T, X ≡ X—X except that cells are trypsinized before their second X.

X—X, T ≡ X—X except that cells are separated by trypsinization after their second X.

X—Surv. ≡ X—X except that a graded series of second doses is given to determine the survival curve.

X—T, Surv. ≡ X—Surv. except that cells are trypsinized before the survival curve exposures.

It should also be noted that, with one exception (fig. 5), in each of the figures to follow the several parts of an overall experiment were performed with the same suspension of cells. For this reason, some of the figures are complex. In each instance, the plating efficiency, PE, (percentage of cells in the initial suspension that grew into colonies) and other pertinent data are noted on the figure. Although we have observed some variation from experiment to experiment in survival parameters even when log-phase cells were used, we have required that the different parts of an over-all experiment performed with the same suspension of cells must be consistent with each other. This is a practice not always followed in work of this type.

Before considering the survival kinetics of cells that have survived a given first dose, it is important to know how unirradiated cells behave. In order to facilitate the analyses, these experiments were performed with cells in log-phase or very close to log-phase growth after their one-hour attachment period. Figure 1 shows the growth and survival kinetics of control cells.[2] The growth curve (open squares) is to be read against the right ordinate and shows that after attachment the population grew exponentially with a doubling time, T_2, of 10.1 hours. The open circles comprise a *Gr.—T, X* experiment since at varying times, the cells in the developing clones were spread by trypsinization, allowed to reattach, and then X-irradiated with a fixed dose of 1065 rad. The closed circles represent a *Gr.—X* experiment; that is the cells were treated in the same way as in the preceding instance except that the developing clones were not trypsinized. By about

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[2] On this figure and those to follow, standard errors in colony counts are shown unless they are smaller than the plotted points.

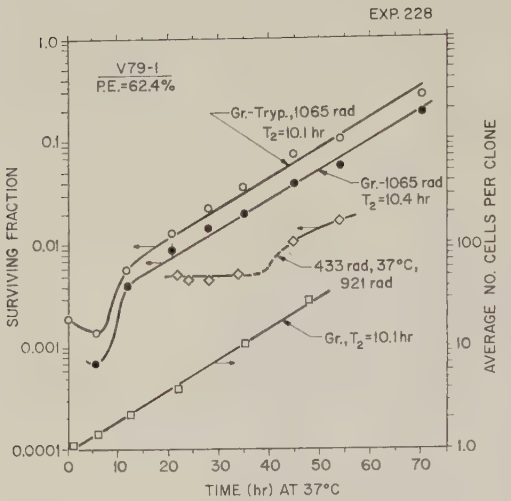

Fig. 1 Variation of survival as a function of time (growth) for cells which received no prior irradiation: open squares, growth curve; open circles, cells trypsinized before receiving 1065 rad; closed circles, cells not trypsinized before receiving 1065 rad. The open diamonds show the net survival to 433 rad followed by 921 rad for varying periods of time between exposures and with no trypsinization treatment.

the end of the first division, both ex
ments show that the survival to a
dose increases in direct proportion to
average number of cells per potentia
ony-forming unit since both T_2's are
sentially equal to that obtained from
growth curve. As will be seen in figu
the *Gr.—X* curve is displaced down
from the *Gr.—T, X* curve, because the
in the former instance were a little
sensitive than in the latter.

Also shown in figure 1 is a typical *X*
experiment (the open diamonds).
their initial attachment period, these
were given a first dose of 433 rad and
the times shown, they all received
same second dose, 921 rad. As the fol
ing figures make clear, by 20 hours
433 rad, surviving cells have repaired
initial sublethal damage and are grow
asynchronously at the same rate as co
cells. The *X—X* curve shows that
though by 20 hours these cells are incr
ing in number exponentially, their sur
to a second dose is essentially constan
more than a generation time and the
creases, in this instance, in roughly a

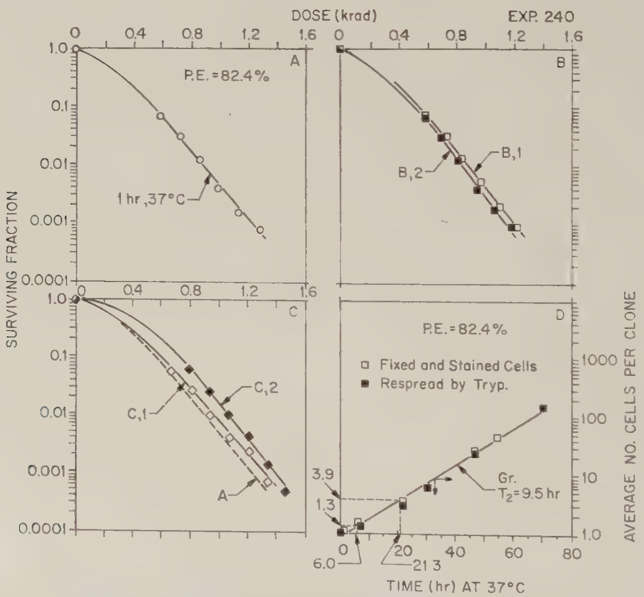

Fig. 2 Survival curves as a function of time (growth) for cells which had received no prior irradiation: *A*, after the 1-hour attachment period; *B*, *1*, after 6 hours of growth with trypsinization before exposure; *B*, *2*, same as *B*, *1*, but no trypsinization; *C*, *1* after 21.3 hours of growth with trypsinization before irradiation; *C*, *2*, same as *C*, *1* but no trypsinization. In panel D, a growth curve is shown determined by two different methods; T_2 is the doubling time. The data is summarized in table 1.

ashion. In view of the *Gr.*—*T*, *X* and
X curves, this kinetics is anomalous
, in essence, the subject of this paper.
ore proceeding with experiments deal-
ith the *X*—*X* anomaly, we would first
o consider the initial dips in the
T, *X* and *Gr.*—*X* curves. We have in-
ated these dips by comparing the sur-
curves obtained after 6 hours of
h with those obtained right after at-
ent, after zero hours, and those ob-
d after about two generation times.
ll become clear from the next figure,
minima at 6 hours result primarily
small decreases in the MLD's of these
compared to either cells at zero time
lls irradiated after their first genera-
of growth.

ure 2 shows the survival curves to
h we have referred. (The survival
are summarized in table 1.) Panel D
s the growth of the cells used in this
cular experiment which, in this in-
e, suffered a brief lag before they
nenced exponential growth. Panel A
s the survival curve[3] after the attach-
of single cells. In panel B, both
s were determined after 6 hours. For
B, *1*, the cells were dispersed by
inization before exposure, and for
B, 2, they were exposed without addi-
l treatment. Therefore, *B*, *1* corre-
ds to a *Gr.*—*T*, *Surv.* experiment and
to a *Gr.*—*Surv.* experiment. These
es show clearly why, at 6 hours in
e 1, the *Gr.*—*T*, *X* point was higher
the *Gr.*—*X* point. In panel C, *C*, *1* is
—*T*, *Surv.* curve and *C*, 2 a *Gr.*—*Surv.*
e both determined after 21.3 hours.
se curves also explain why in figure 1
Gr.—*T*, *X* data was higher than the
–*X* data throughout the region of
th in which survival increased in proportion to the average number of cells per potential colony-forming unit (see table 1).

It is also instructive to compare the over-all extrapolation numbers, $n\overline{N}$, at the three times shown. For trypsinized cells—panel A and curves *B*, *1* and *C*,*1*—the $n\overline{N}$ values are about the same; 5.9, 5.4, and 5.1 respectively. (Since the average cellular multiplicity in these instances is very close to 1.0 ($\overline{N} \cong 1.0$), the value of $n\overline{N}$ is essentially the average single-cell extrapolation number.) At 6 hours, panel B, the $n\overline{N}$ for *B*, 2 is 7.4 which is 1.4× that for *B*, *1*. From panel D, the growth factor at 6 hours is 1.3. At 21.3 hours, panel C, the ratio of the $n\overline{N}$ values is 3.9 which agrees with the growth factor shown in panel D. Curve *A* is redrawn in panel C since the MLD's for curves *A* and *C*, 2 are equal making it more apparent that in the exponential regions of both curves, *C*, 2 is uniformly displaced upward from *A* by just about the factor of growth.

In summation, the points contained in the first two figures with respect to the survival properties of control cells are the following: (1) Except for about the first generation of growth, the survival of cells in active growth increases in direct proportion to the average number of cells present whether these cells are separate or in clones at the time of exposure. This means that the MLD's are constant and that the over-all extrapolation number increases in direct proportion to growth. (2) During

TABLE 1

Survival parameters for data in figure 2

Curve	Hours	P.E.	Exp. 240	
			nN̄	MLD
		%		rad
A	0	82.4	5.85 ± 2.02	140.8 ± 13.6
B, *1*	6.0	81.6	5.41 ± 0.53	139.8 ± 4.1
B, *2*	6.0	82.4	7.44 ± 3.06	127.7 ± 15.2
C, *1*	21.3	98.4	5.08 ± 0.53	153.5 ± 4.5
C, 2	21.3	82.4	20.0 ± 5.8	141.0 ± 10.6

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[3] The extrapolation numbers, MLD's, and their uncertainties were determined from least square analyses assuming that the portions of the survival curves starting at about 10% of the control surviving fractions were exponential. We are indebted to Dr. Mones Berman for utilizing his computer program for the National Bureau of Standard's IBM 704 computer for this purpose.

the first generation of growth, the sensitivity of the cells as individuals or in clones goes through a maximum so that the survival for a fixed dose goes through a minimum. (This variation in sensitivity may be more apparent than real if it is principally a reflection of shape changes as rounded cells become flattened during the first several hours.) The over-all extrapolation number, however, still appears to increase in direct proportion to growth although a small lack of proportionality cannot be ruled out. And (3) consequently it can be concluded that control cells survive independently whether or not they are in clones when irradiated. This last point is true from at least the 2 to the 100-cell-per-clone stage and is probably true from the single to the 100-cell-per-clone stage.

Anomalous survival due to fractionation

We will now show two of our early experiments which, in the light of our more recent results, probably displayed anomalous fractionation survival curves; that is anomalous *X*—*X* curves. Figure 3 shows an initial survival curve (closed circles), and an *X*—*X* curve (open squares) performed with clone *A* Chinese hamster cells. The experiment was started with single cells

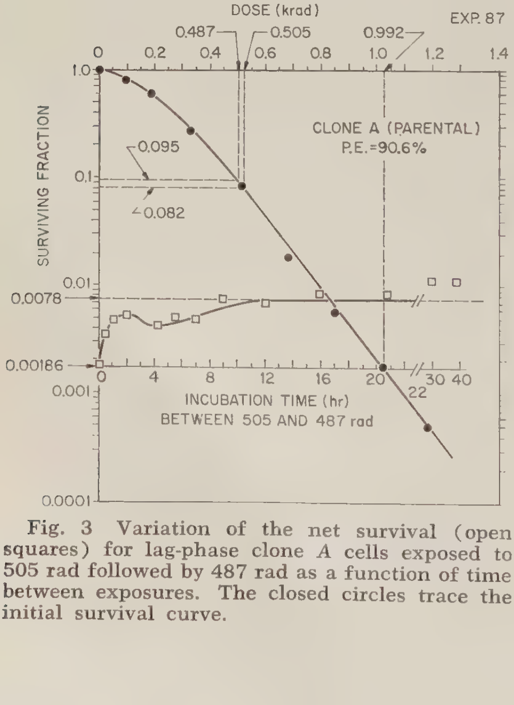
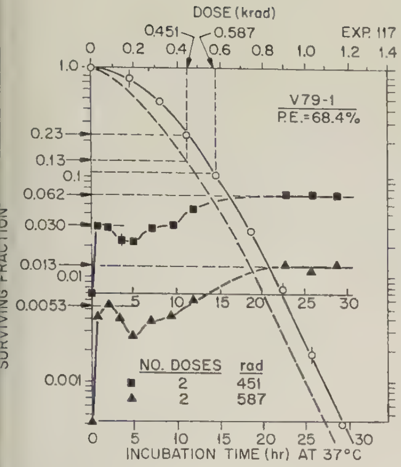


Fig. 3 Variation of the net survival (open squares) for lag-phase clone *A* cells exposed to 505 rad followed by 487 rad as a function of time between exposures. The closed circles trace the initial survival curve.

which were initially in lag phase. Fo
fractionation curve, the doses were 50
followed by 487 rad. The survival c
shows that if the total dose, 992 rad,
given at one time—during a period o
minutes—the net survival would have
0.0019. This is plotted on the left ordi
at zero hours. As the time between d
was increased, the net survival incre
in an oscillating manner. From abou
to 22 hours, the net survival was cons
In a separate experiment with clon
cells, we showed (Elkind and Sutton,
that by 18 hours after 505 rad the curv
surviving cells returned to the shap
the initial survival curve suggesting, th
fore, that the entire *X*—*X* plateau c
sponded to the complete repair of suble
damage. Since control cells survive i
pendently, the plateau also suggested
from 10 to 22 hours recovered, survi
cells did not divide. If they had, in ana
with control cells, we would have expe
the net survival to have increased in a
that would have reflected the increas
the average cellular multiplicity per
tential colony-forming unit. In this exp
ment, we did not have an indepen
measure of the total division delay (gro
lag plus radiation-induced lag). As a
sult, we felt that the data could be c
sistently interpreted if we assumed t
division did not start until about 22 ho
after the first exposure. In retrospect,
believe that division probably sta
around the beginning of the plateau
that the plateau itself is anomalous.

In the clone *A* experiment, the cont
population was in lag phase at the start
the experiment. Figure 4 shows two fr
tionation experiments performed with
same suspension of log-phase cells. T
is a second illustration of what proba
represents anomalous fractionation s
vival. In this case, the first exposures w
performed 16 hours after an initially l
phase population of *V79-1* cells w
plated. From other measurements,
knew that by 16 hours after plating, th
cells were in log-phase growth and h
undergone at least a two-fold increase
number. Detailed analyses of both *X*-
curves (Elkind and Sutton, '60) indica
that in these experiments the plate
were at survival levels twice that co



4 Two recovery curves for log-phase
cells starting with an average cellular
plicity of 2.0 to 2.5: Closed squares, the net
al to two 451 rad doses as a function of time
en exposures; closed triangles, two 587 rad
versus time. The open circles trace the
survival curve at zero time (16 hours after
g).

ding to full recovery. Because this
riment was started when the cells were
ing exponentially, it is reasonable that
0 hours after a first exposure, surviv-
ells had completed one division. How-
if the progeny of surviving cells sur-
like control cells, from 20 hours on,
X—X survivals should have increased
a doubling time of about 11 hours.
efore, in retrospect once again, the
aus in these experiments are probably
nalous.

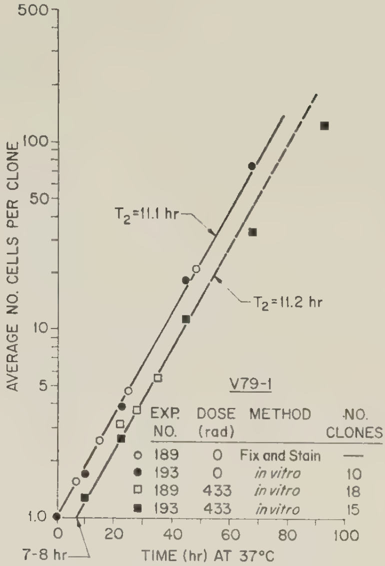


Fig. 5 Growth curves of unirradiated cells and log-phase cells which had survived 433 rad: open circles, by counting fixed and stained cells; closed circles, by following the growth of specific cells using phase microscopy; open and closed squares, same as closed circles but for irradiated cells. T_2 stands for the doubling time.

Postirradiation growth of surviving cells

o initiate our study of the kinetics of
iving cells, we first learned how to
e single-cell suspensions (average
iplicity less than 1.1) of log-phase
so that contributions to postirradia-
growth lags could be minimized. This
ed out to be easy to do since we found
suspensions made of cells in early log
se, when replated, resumed exponential
vth with small, if any, delays (fig. 5).
open circles represent a growth curve
rmined by counting the cells in clones
l and stained as a function of time.
closed circles refer to cells from an-
r suspension whose growth was determined by a method we call *"in vitro"* counting. Single cells were allowed to attach in optically flat petri dishes containing growth medium. They were then located with respect to the stage coordinates of an inverted microscope equipped with phase optics (magnification 100× to 150×). These dishes were returned to the incubator, and as a function of time, the numbers of cells at the zero time coordinates were counted. By following the developing clones until they reached about the 100-cell-per-clone stage, we could then identify, with considerable certainty, which of the initially located single cells were viable. The growth curve constructed from this data agrees with that obtained from counts of fixed and stained cells (and with curves obtained by trypsinization procedures used in other experiments) and is largely free from any ambiguities connected with plating efficiencies less than 100%.

From a number of considerations we chose to follow the postirradiation kinetics of cells exposed to a dose of 433 rad. (Data after smaller doses indicate that the postirradiation kinetics after the onset of division is closer to that obtained with cells which received no prior irradiation.) A dose of 433 rad corresponds to approximately 20% survival and, as can be seen in figure 5, often induced a delay of about ¾ of a generation time. The open and closed squares are *"in vitro"* counts of the growth of surviving cells after 433 rad. These data come from two different experiments one of which, experiment 193, was the same experiment in which the *"in vitro"* control counts were made. It is apparent that surviving cells grew asynchronously and with a doubling time equal to that of the controls. Although a number of the delays measured in other experiments were close to 7–8 hours, values of 4 and 10 hours have been observed. The use of the *"in vitro"* scoring technique for surviving cells largely obviates concern with cells of limited proliferative ability induced by the exposure, or which may have been present in the control suspension. In figure 5, the open squares which came from experiment 189 should be noted. These points lie on the surviving cell growth curve and will appear again in figure 7.

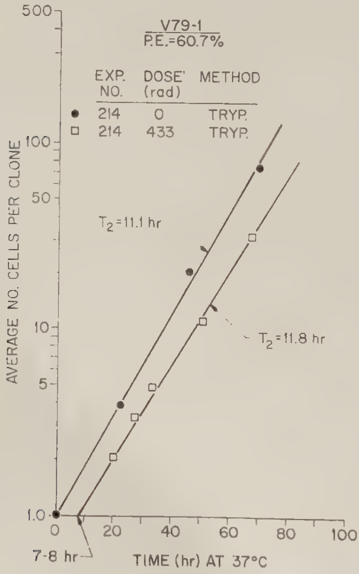


Fig. 6 Growth curves for unirradiated cells (closed circles) and log-phase cells which have survived 433 rad (open squares). Growth was determined by trypsinizing developing clones as a function of time so that each cell could grow into a visible colony. T_2 stands for doubling time.

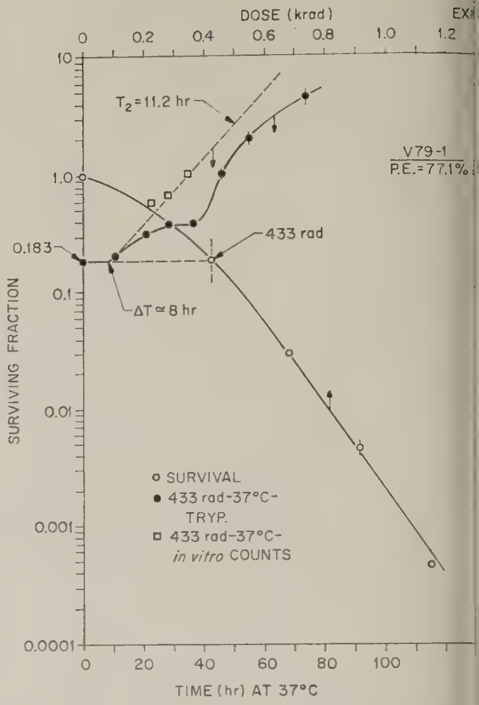


Fig. 7 An apparently anomalous growth c
after 433 rad: open circles, the initial surv
curve; closed circles, growth after 433 rad d
mined by trypsinization as a function of t
open squares, the same data plotted in figu
determined by counting the number of cell
specific clones as a function of time. The
methods of determining growth were perfor
with cells from the same initial suspension
stands for the doubling time determined from
data in figure 5.

Our more frequently used method of
termining growth curves is shown in fig
6. As a function of time, control and
radiated cells were spread by trypsinizat
and then the dishes containing them
turned to the incubator to permit
growth of viable single cells into vis
colonies. For control cells (closed circle
this is what we mean by a *Gr.—T* cur
for irradiated cells, an *X—T* curve.
determination of growth curves using
trypsinization technique is considera
easier experimentally than the *"in vi*
scoring method and has usually yielded
sults consistent with those obtained w
the latter method.

While almost all of our *X—T* cur
have shown that survivors grow asynch
nously and as rapidly as control cells,

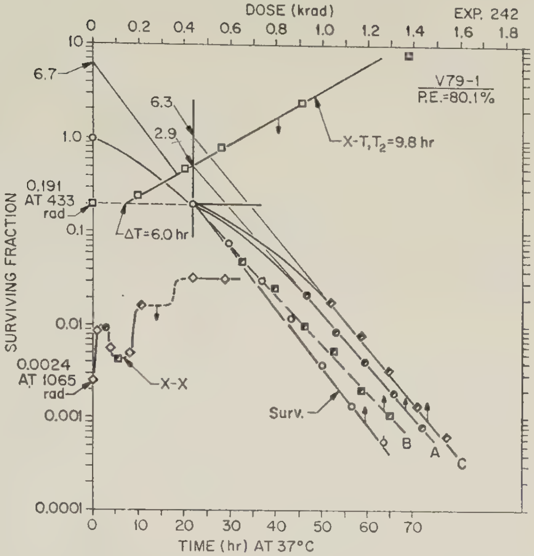


Fig. 8 Recovery and fractionated survival curves for log-phase cells: *Surv.*, the initial survival curve after a 1-hour attachment period; *A*, survival curve 2.6 hours after 433 rad; *B*, survival curve 5.3 hours after 433 rad; *C*, survival curve 10.6 hours after 433 rad; *X—T*, growth curve determined by trypsinization ($T_2 =$ doubling time); *X—X*, net survival to 433 rad followed by 632 rad as a function of time between doses. The data are summarized in table 2.

observed a few exceptions. One of e is shown in figure 7 (another will ear in figure 9). The survival curve is cated by the open circles. The *X—T* e is traced by the closed circles. The nsistency between the *X—T* growth e and the *"in vitro"* counts could have lted from either of these two possibil- . (1) Since the procedure used in the *T* experiment was to make a trypsi- d suspension at the times shown from ch aliquots were inoculated into fresh es, the ability of trypsin to remove cells n the glass surfaces of their original es may have varied with time. (2) rnatively, the cells may have been re- ed adequately from the glass surfaces, the ability of trypsin to separate them have depended on when the cells were sinized. At the present time, we have vay of choosing between these alterna- s, although in our experience we have er had reason to doubt that trypsin did strip off essentially all of the cells at- ed to a glass surface. In addition, as strated by figure 8, the roughly stepwise pe of the *X—T* curve is similar, tem- lly, to the stepwise variation of the over-all extrapolation number of surviving cells and their progeny. The *X—T* curve does *not* indicate, however, asynchronous division at the onset of division at about 8 hours.

Postirradiation variations in extrapolation number

To this point we have described data which shows that: (1) control cells survive independently; (2) in contrast to control cells, surviving cells that have fully recovered, survive in a manner which may in part be independent of postirradiation population growth; and (3) after a dose of 433 rad, surviving cells usually suffer a delay of about ¾ of a generation time and then increase in number at the same rate as control cells. We will now describe several experiments which show that after recovery, the survival properties of *surviving* cells are indeed anomalous in comparison with control cells in that the overall extrapolation number, $n\overline{N}$, does not increase in direct proportion to the average cellular multiplicity, $\overline{N}$.

Figure 8 (see table 2) shows the first of these experiments, all of which were per-

TABLE 2

Description of curves and survival parameters for data shown in figure 8

Curve	Description	Exp. 242	
		$n\overline{N}$	MLD
			rad
Surv.	Initial survival	6.74±0.97	134.5±5.2
A	433 rad; 37°C., 2.6 hrs.; Surv.	2.9	155
B	433 rad; 37°C., 5.3 hrs.; Surv.	1.0	165
C	433 rad; 37°C., 10.6 hrs.; Surv.	6.28±0.89	147.2±6.2
X—T	433 rad; 37°C., trypsinization		
X—X	433 rad; 37°C., 632 rad		

formed starting with a single cell suspension of cells which subsequently grew exponentially with a small, if any, delay. The initial survival curve is shown by the open circles. The doses used in the *X—X* experiment were 433 rad followed by 632 rad. The curves labeled *A*, *B*, and *C* are *X—Surv.* curves. The point at 2.6 hours after 433 rad (half closed circle) was taken from curve *A;* the point at 5.3 hours (half closed square) from curve *B*; and the point at 10.6 hours (half closed diamond) from curve *C*. The shape of the dashed portion of the *X—X* curve was inferred from other experiments. This latter region of the *X—X* curve, as well as the plateau follows it, was not as flat as shown i experiments due probably to small osc tions in the MLD of the over-all surv curve during the growth of surviving c

The *X—T* curve in figure 8 shows surviving cells did not start to divide 6 hours after 433 rad in this experim Therefore the portion of the *X—X* curv to 6 hours represents the response of si cells. The initial survival curve indic that immediately after 433 rad, the ex polation number of surviving cells close to 1.0. By 2.6 hours, curve *A* sh that $n\overline{N}$ has returned to about half its

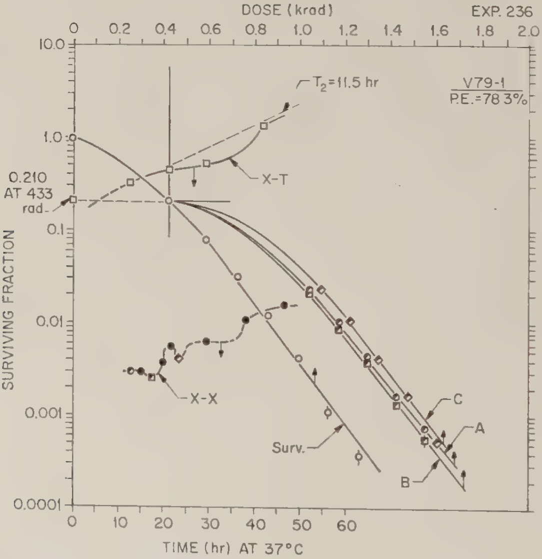


Fig. 9 Recovery and fractionated survival curves for log-phase cells: *Surv.*, initial survival curve after a 1-hour attachment period; *A*, survival curve 13.2 hours after 433 rad; *B*, survival curve 17.6 hours after 433 rad; *C*, survival curve 23.7 hours after 433 rad; *X—T*, growth curve after 433 rad determined by trypsinization as a function of time; *X—X*, net survival to 433 rad followed by 921 rad as a function of time between doses. The data are summarized in table 3.

TABLE 3

Description of curves and survival parameters for data shown in figure 9

urve	Description	Exp. 236	
		$n\bar{N}$	MLD
			rad
urv.	Initial survival	9.48±0.84	127.6± 3.5
A	433 rad; 37°C., 13.2 hrs.; Surv.	7.49±0.91	146.7± 5.1
B	433 rad; 37°C., 17.6 hrs.; Surv.	9.08±1.85	137.5± 8.1
C	433 rad; 37°C., 23.7 hrs.; Surv.	17.2 ±6.9	134.2±14.5
—*T*	433 rad; 37°C., trypsinization		
—X	433 rad; 37°C., 921 rad		

value, and by 5.3 hours, $n\bar{N}$ has ped back to 1.0. (For curves *A* and *B*, *n* since $\bar{N} \cong 1$.) Hence, just before ion starts, one event appears to be suf- t to kill a cell surviving the first dose ugh the sensitivity of these cells is less than it was initially.[4] By the time verage multiplicity of surviving cells, as increased to 1.4, at 10.6 hours, the polation number has returned to its l value. From analogy with control one might have expected the net sur- from about this time on to have in- sed in direct proportion to the aver- multiplicity. This did not happen.

e format of figure 9 (see table 3) is ame as in figure 8 except that the re- from about 13 hours through the letion of the first division is presented. egin with, the *X—T* curve in this ex- ment is another illustration of the in- ently observed postirradiation growth referred to earlier. For comparison the much more usual situation, a ed line with a T_2 of 11.5 hours is n. On the *X—X* curve, the points at 17.6, and 23.7 hours (the half-closed ols) were obtained from the *X—Surv.* s at the corresponding times which abeled *A*, *B*, and *C* respectively. From nspection of the over-all extrapolation bers, $n\bar{N}$, it is seen that from about 13 3 hours, $n\bar{N}$ equals the initial value that by 24 hours, $n\bar{N}$ has increased by t a factor of 2. Also, the oscillations e *X—X* curve in this case can be seen due most likely to small oscillations he MLD's of the *X—Surv.* curves points at the minima came from es *B* and *C* whose MLD's are both ler than the MLD of curve *A*. The data in this figure show that by about the time surviving cells have all divided once, the over-all extrapolation number has increased by about a factor of 2.

An examination of the region of the second plateau in the *X—X* curve is shown in figure 10 (see table 4) whose format is similar to the two preceding figures except that the *X—Surv.* curves have their origins plotted at the times corresponding to when they were performed. The dose scales in each instance are the same but displaced to the right. This was done because if they had all been plotted from the same origin (e.g. the 433 rad point on the initial survival curve), they would have appeared indistinguishable. *X—Surv.* curves *A*, *B*, and *C* were determined at 20.1, 27.1, and 34.4 hours. During this interval of time, the average multiplicity of surviving cells had increased by a factor of 2.4. The over-all extrapolation numbers of *A*, *B*, and *C* are equal and these are all larger than the initial extrapolation number by about a factor of 2. From the *X—T* curve, it can be seen that by 20 hours the first division has been completed. The small uncertainties on the values of $n\bar{N}$ clearly show that the over-all extrapolation number did not increase in proportion to growth in the 14-hour interval examined, and because the MLD's are equal, the three *X—X* curves—curves *D*, *E*, and *F*—also illustrate this fact. In summary, this figure shows that from the end of the first generation time until at least three hours beyond the end of the second generation time, the

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[4] It should be noted that the MLD's for curves *A* and *B* may be larger than those observed if surviving cells experience a similar variation in MLD resulting from morphological changes during the first several hours as do cells which received no prior irradiation (see figures 1 and 2).

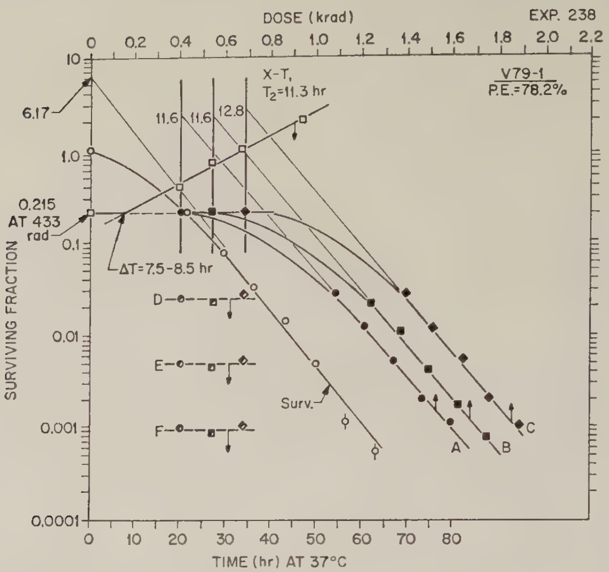


Fig. 10 Recovery and fractionated survival curves for log-phase cells: *Surv.*, initial survival curve after a 1-hour attachment period; *A*, survival curve 20.1 hours after 433 rad; *B* survival curve 27.1 hours after 433 rad; *C*, survival curve 34.4 hours after 433 rad; *X—T* growth after 433 rad determined by trypsinization as a function of time (T_2 is the doubling time); *D, E,* and *F* are the net survivals to 433 rad followed by 698, 948, and 1198 rad respectively as a function of time. To avoid overlapping curves, the origins of curves *A, B,* and *C* are plotted at the times that they were determined. In each instance, the dose scale is the same as that of the initial survival curve, but displaced to the right according to the time scale shown. The data are summarized in table 4.

TABLE 4

Description of curves and survival parameters for data shown in figure 10

Curve	Description	Exp. 238	
		nN̄	MLD
			rad
Surv.	Initial survival	6.17±1.52	139.5±11.6
A	433 rad; 37°C., 20.1 hrs.; Surv.	11.6 ±2.09	152.8± 8.8
B	433 rad; 37°C., 27.1 hrs.; Surv.	11.6 ±0.63	150.2± 2.8
C	433 rad; 37°C., 34.4 hrs.; Surv.	12.8 ±0.95	152.6± 3.6
A, B, C	Data treated as 1 curve	11.6 ±3.4	153.2±12.5
X—T	433 rad; 37°C.; trypsinization		
D	433 rad; 37°C., 698 rad		
E	433 rad; 37°C.; 948 rad		
F	433 rad; 37°C.; 1198 rad		

over-all extrapolation number appears to have been constant. The value of $n\bar{N}$ during this interval is about twice that for the initial single-cell population. Therefore, at 34.4 hours, on the average 5 cells survive with the same probability as two cells. Further, this probability, aside from small changes in MLD's, is about twice as large as that of single log-phase cells.

Apparent survival dependence

In comparison with control cells, interpretation of the stepwise variatio the over-all extrapolation number is after recovery, the progeny of survi cells do not survive independently. is, when a population of clones is irradi for the second time, damage can be sh by cells in close proximity, so that

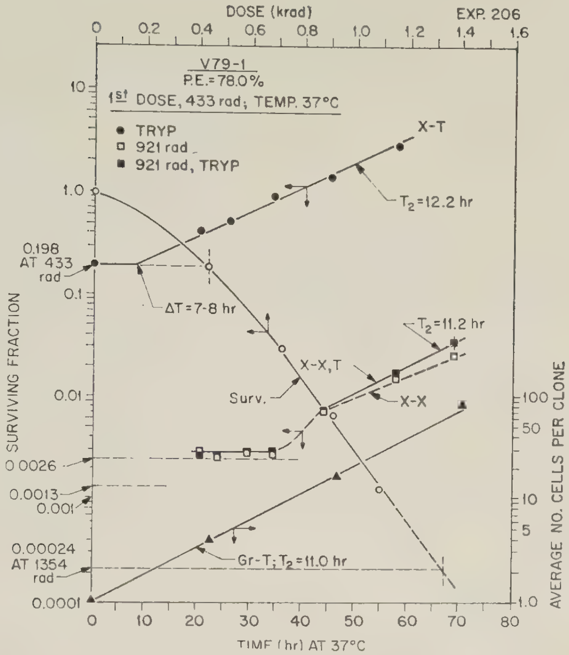


Fig. 11 The effect of spreading cells in clonal growth after their second exposure: *Surv.*, he initial survival curve; *Gr.—T*, growth of control cells by trypsinization as a function of ime; *X—T*, growth after 433 rad by trypsinization as a function of time; *X—X*, net survival o 433 rad followed by 921 rad as a function of time; *X—X, T*, same as *X—X* except that the ells in developing clones were dispersed by trypsinization immediately after the 921 rad xposure. T_2 stands for doubling time.

ability of survival of a clone does not
ase in proportion to the average num-
of viable cells per clone or the cellular
iplicity. We have tested for this pos-
ty by performing a series of experi-
ts in which cells in developing clones
separated by trypsinization before
after their second exposure. Because
lisparity between growth and over-all
apolation number was larger from 20
s on, we confined our examination to
region of the second plateau.
the experiments shown in figure 11,
tudied the effect of separating the cells
their second exposure. To begin with,
Gr.—T* curve shows that the cells used
is experiment grew exponentially after
ng. The *X—T* curve shows that cells
iving 433 rad suffered a delay of 7–8
s and then also grew asynchronously.
open circles trace the initial survival
e. In the *X—X* experiment the two
s used were 433 rad and 921 rad. For
oses of orientation, on the left ordi-
three survival levels are indicated. If the MLD of surviving cells remained equal to its initial value and if there had been no recovery, the net survival of single cells after 1354 rad would have been 0.00024. If there had been full recovery between doses and no division, the net survival would have been 0.0013. And if there had been full recovery and one division with both cells surviving independently, the survival level would have been 0.0026. The *X—X* curve shows that by 20 hours the survival corresponds to the situation described last but that for the next 14 hours, the net survival remained constant. The *X—X, T* curve (i.e., cells separated by trypsinization after their second exposure) shows that if the cells are separated within 15 minutes after their second exposure, there is no effect on the survival probability in the flat region. Hence, it is clear that if there are any coupling effects between adjacent cells in a developing clone, these effects are fixed at about the time of exposure and are not related to events that might occur during the succeeding 12

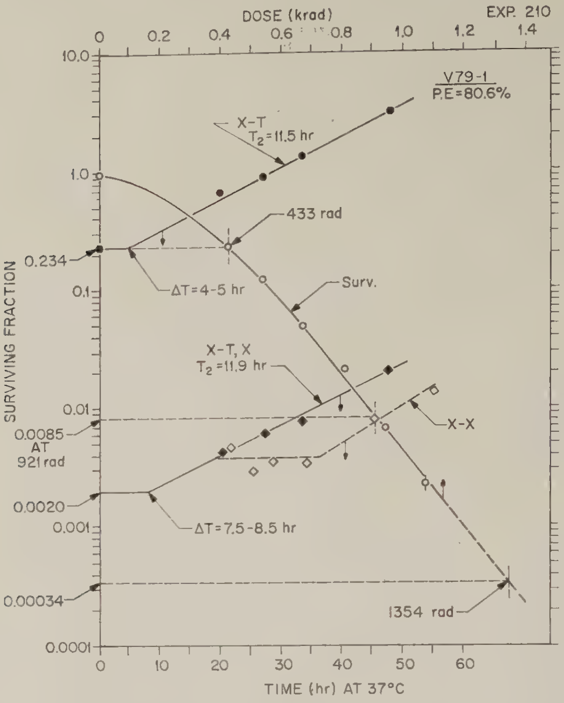
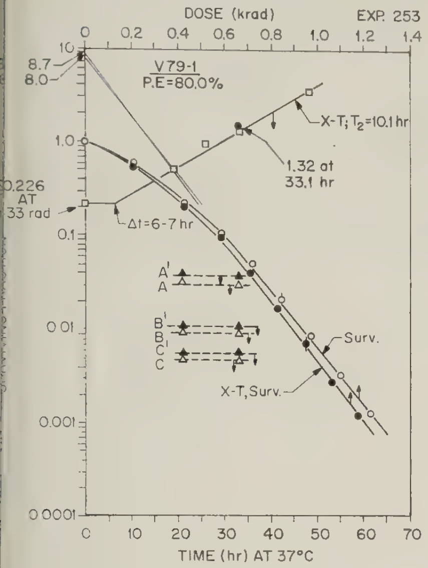


Fig. 12 The effect of spreading cells in clonal growth before their second exposure: *Surv.*, the initial survival curve; *X—T*, growth after 433 rad determined by trypsinization as a function of time; *X—X*, net survival to 433 rad followed by 921 rad as a function of time; *X—T, X*, same as *X—X* except that the cells in developing clones were dispersed by trypsinization at the times shown, allowed to reattach for 1 hour, and then given their second exposure of 921 rad. T_2 stands for doubling time.

days required for the growth of surviving cells into visible colonies.

Figure 12 shows an experiment similar to the last one except in this case the cells were trypsinized before the second exposure. The reference survival levels are: 0.00034 for the net survival without recovery; and 0.0020 for full recovery without division. The *X—T* curve shows that the survivors grew exponentially. The *X—X* curve (open diamonds) shows a small wiggle between 20 to 27 hours probably due to a small oscillation in the MLD during that interval. However, the *X—T, X* experiment shows that the net survival increased in direct proportion to growth. It should be noted that, in the *X—T, X* experiment after trypsinization, the dishes were placed at 37°C. for one hour to permit the cells to reattach to their glass surfaces. Taken at their face value, the data in figures 11 and 12 suggest that during the second and part of the third gene tion times after the onset of growth, progeny of surviving cells can share th damage only if they are in contact w each other. For cells that display a fib blast-like morphology when attached glass, as these cells do, this contact can quite intimate in clonal growth.

As a further check on the aforegoing terpretation, the experiments shown in ure 13 were performed (see table Parts of three *X—X* and *X—X, T* cur were determined which are labeled: *A* and *C*; and *A′*, *B′*, and *C′* respectively. this experiment, the *X—X, T* curves are slightly above their corresponding *X*- curves, but nevertheless it is still clear t trypsinization after the second expos did not relieve any appreciable coupl that might have existed between cells. the *X—T, Surv.* curve the trypsinizat was performed at 33.1 hours after 433 e cells were given their second doses wing a one-hour attachment period. control survival point for this curve o plotted along the X—T curve (closed) and, therefore, is relative to the initial control for the whole experiment. The extrapolation number of the X—T, *Surv.* curve equals that of the initial survival curve. On the other hand, if at 33 hours the progeny of surviving cells left in close proximity survived independently, their average extrapolation number per cell would have had to drop to about 2.8. That is the initial extrapolation number, which was 8.0, times 2 (see figures 8 through 12) divided by the average viable cell multiplicity at 33 hours which was 5.8. In view of the fact that the extrapolation number for single cells at 33 hours equals the initial value, it is quite unlikely that the single cells obtained by trypsinization at 33 hours survived in the same way as nontrypsinized cells if the latter cells had survived independently (see figure 14).



. 13 The effect of spreading cells in clonal th before and after their second exposures: , the initial survival curve; X—T, *Surv.*, the val curve (normalized to 1.0) of cells which trypsinzied 33.1 hours after 433 rad and allowed to reattach for 1 hour before their sures; X—T, growth after 433 rad as a func- of time (the solid circle represents the sur- of the control for the X—T, *Surv.* curve rela- to the control for the X—T curve, 1.0); A, B, C, net survivals to 433 rad followed by 722, and 1020 rad respectively; A', B', and C', as A, B, and C except that after the second s, the cells were trypsinized. T_2 stands for the ling time. The data are summarized in 5.

DISCUSSION

Figure 14 shows a schematic representation of the variation in the over-all extrapolation number, $n\overline{N}$, as a function of time after 433 rad. As already noted, there are also significant variations in sensitivity, MLD, but these usually amounted to 10% or less for times longer than the division delay period, Δt. The abscissa is time in units of the doubling time, T_2 of surviving cells after their division delay.

From different experiments, some of which have been presented in this paper, we know that the initial extrapolation number of single control cells, n_c, varies from experiment to experiment. In one instance, it was clearly higher than the value found in the region of the first plateau. We believe that this variation may be due to two things: (1) The initial ex-

TABLE 5

Description of curves and survival parameters for data shown in figure 13

Curve	Description	Exp. 253	
		$n\overline{N}$	MLD
			rad
Surv.	Initial survival	8.00 ± 1.13	141.4 ± 4.1
X—T. *Surv.*	433 rad; 37°C.; 33.1 hr.; trypsinization, Surv.	8.65 ± 0.41	133.1 ± 0.7
X—T	433 rad; 37°C.; trypsinization		
A	433 rad; 37°C.; 722 rad		
A'	433 rad; 37°C.; 722 rad, trypsinization		
B	433 rad; 37°C.; 921 rad		
B'	433 rad; 37°C.; 921 rad, trypsinization		
C	433 rad; 37°C.; 1020 rad		
C'	433 rad; 37°C.; 1020 rad, trypsinization		

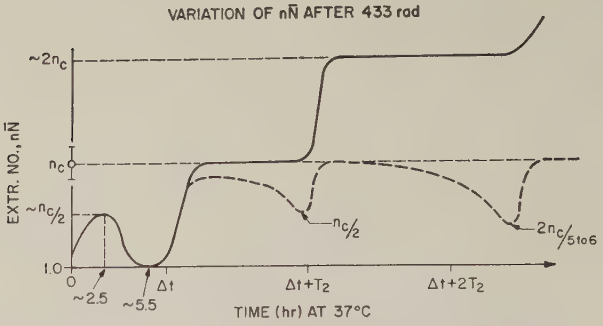


Fig. 14 Schematic summary of the variation in the over-all extrapolation number, $n\bar{N}$, as a function of time after 433 rad. The solid line up to the onset of division, Δt, is for single cells; the dashed line represents the variation of the extrapolation number for single cells if they survived independently; n_c is the initial single cell extrapolation number. T_2 stands for the doubling time of surviving cells after the onset of division.

trapolation number may reflect a characteristic of the starting population which is, to some extent, independent of its growth phase at the start of an experiment. And (2) we have consistently observed, with different cell lines, that the initial extrapolation number varies inversely with the MLD. Our most consistent results, in terms of the kinetics sketched in figure 14, were obtained when variations in the MLD throughout an experiment were 10% or less. In the instance in which $n\bar{N}$ in the region of the first plateau was clearly less than n_c, the MLD was 27% larger than for the control cells, and in another experiment, it was 40% larger than usually found for the starting population. In this connection, it is worth noting that in figure 8, the extrapolation number dropped to 1.0 at 5.3 hours (the minimum in figure 14) and that the MLD at that time was 23% greater than the MLD of the starting population. In view of these considerations, and our present state of ignorance with regard to the interrelationship between extrapolation number and sensitivity, n_c is shown with some uncertainty.

A first dose of 433 rad reduces the initial survival to close to the region of exponential survival. Therefore, at zero time after 433 rad, the over-all extrapolation number is close to 1.0. In about ⅓ to ½ of the division delay period, $n\bar{N}$ increases to about $n_c/2$ and returns to close to 1.0 just before the onset of division. Up to the time Δt, therefore, the extrapolation number observed is for single cells. Within the first half of the first division cycle, increases to n_c and remains fairly c stant until about the beginning of the s ond division. It then increases by abou factor of 2 and remains constant for th to 4 hours beyond the end of the sec division cycle. In spite of these variatio in $n\bar{N}$, from time Δt on, surviving c grow exponentially. After initial doses l than 433 rad, the second plateau may absent; that is, during the first divis after Δt, $n\bar{N}$ may start to increase in p portion to growth.

There are at least two explanations parts of these findings. The first one based upon a literal interpretation of of the experiments presented but leads a conclusion somewhat at variance w some generally accepted ideas. To c sider the second interpretation, it is nec sary to ignore some of our data. The sult, however, is a view which does challenge, in any serious way, any of ideas and leads to some insights which m prove to be of value.

The first interpretation is concerned marily with the plateaus in the $n\bar{N}$ cu and is based mainly on the data obtai from the beginning of the second pos radiation division cycle. Our data sho that the over-all extrapolation number constant in this region for cells in clo association at the time of their second posure or for cells separated shortly the after. If the cells are separated by trypsi zation one hour before their sec exposure, they survive in a way that

ts the fact that their number has in-
ased by about 2.5× in this region.
ce in one hour, cells in log-phase growth
uld have been expected to have in-
ased in number by only 7%, the con-
sion follows that recovered cells can
nsmit damage between themselves in
regions of the plateaus. In terms of
get theory, this means that the sites of
primary ionizations may be removed
m the sites of the principal effects by at
st two cell walls and possibly two nu-
ar membranes as well.

The foregoing interpretation offers no ex-
nation for the events that occur during
period of division delay nor for the
id increase in over-all extrapolation
mber that occurs at about the end of
first postirradiation division. Also,
ce it is based upon the exponential por-
ns of survival curves, it remains a pos-
ility that the shoulder regions of these
ves may result from a different inacti-
ion mechanism which, during the
urse of postirradiation growth, does not
d to the coupling effects inferred.

The observation essential to the first in-
pretation is that cells separated before
ir second exposure survive independ-
tly as do cells that received no prior ir-
diation whether separated or not. Left
gether at the time of their second expo-
re, the progeny of surviving cells appear
survive in a way dependent upon their
ximity to each other. Since trypsiniza-
n was used to spread the cells, it is pos-
le that this, or the one-hour attachment
riod that followed, caused these cells to
rvive with an extrapolation number
ual to that of control cells. That is, it is
ssible that the progeny of surviving cells
survive independently but that their
gle cell extrapolation numbers undergo
ctuations in a manner traced approxi-
ately by the thick dashed line in figure
. If this was the case, at the end of the
st division, the average extrapolation
mber for single cells would have been
the control value and at the end of the
cond plateau, 2/6 to 2/5 of the control
lue. After this period, the extrapolation
mber per cell returns to the control
lue, although some of our results indi-
te there may have been additional fluctu-
ions.

If it is assumed, therefore, that the trypsinization procedure before the second dose introduced an artifact, the essential feature of the second interpretation is that as cells terminate their division delay, they assume different values of extrapolation number per cell so that the value for the over-all population appears to be constant for most of the first division cycle. At the start of the second division, the variation in extrapolation number per cell is repeated, but since there are twice as many cells, the over-all value is twice as large. The variation of the extrapolation number during the period of division delay could then be viewed as due to the combined effects of recovery plus the progression of cells toward division. The initial rise in the curve would represent recovery, and the subsequent fall, the movement of cells toward division. This suggests further that the control extrapolation number might reflect the over-all influence of a number of submoieties. Although the survival parameters, aside from recovery, might have different distributions before and after exposure, it is quite possible that a mixture of various extrapolation numbers and MLD's could yield an over-all survival curve starting with a negative slope and appearing to represent an homogeneous population within the resolution of our experiments.

While this second interpretation does not involve intercellular effects, it is not entirely consistent with our data. To begin with, the second plateau extends for a significant length of time beyond the end of the second division cycle. Also, if the division delay is the result of a mitotic block so that cells accumulate just prior to division, then the subsequent growth would be expected to be synchronous and not asynchronous as we have observed.

However, while the first interpretation applied to the recovery of cells is difficult to reconcile with a genome hypothesis based upon a target-theory model which precludes significant interactions between DNA structures and other parts of the cell, the second interpretation does not rule out a target-type of model providing that the number and/or sensitivity of the targets is dependent upon the age of a cell with respect to its interdivisional cycle. Clearly, more information is needed before a choice between these alternatives can be made or before another model can be favored.

OPEN DISCUSSION

Revesz[5]: In experiments designed to study the clonal survival of X-irradiated cells *in vitro,* little attention has been given so far to the possible effect of the presence or absence of oxygen in the medium during irradiation. The tissue culture technique used by Drs. Elkind and Puck implies that cells have ample access to oxygen in the course of irradiation. Astonishingly enough, with the exception of a single publication (D. Dewey: Nature, *186:* 780, '60) no reports have yet appeared on experiments in which the survival of mammalian cells was studied *in vitro* after irradiation under anoxic conditions. In cooperation with Dr. J. E. Robinson at the Radiology Department of Stanford University we performed experiments to study the clonal survival of several mammalian cell lines after irradiation in the absence of oxygen, substituting air with nitrogen during X-ray treatment. In summary it would seem that cells irradiated in anoxia have (1) decreased radiosensitivity as compared to the sensitivity in air, and (2) the extrapolation number of the survival curves is always *1,* in contrast to the situation found in the presence of oxygen.

Elkind: Do I understand correctly that the extrapolation number equals *1* with and without oxygen in your particular case, or just without oxygen?

Revesz: Without oxygen the extrapolation number is *1,* with oxygen it is higher, and consequently, a shoulder is apparent as you found also in your experiments.

Elkind: How large is the extrapolation number?

Revesz: In air it is between 2 and 3.

Elkind: This is an interesting finding. It does not agree, however, with the results of Dr. Dewey or with the results of other people. That is, they find extrapolation numbers, even under anoxic conditions, which are greater than *1* although the extrapolation numbers with and without oxygen may not be equal.

Powers[6]: I should like to raise a question concerning the criteria. Recall that in the irradiated bacterial spore in the dose-rate ranges used—the germination proc is not affected at all; the cells that unde one division do not necessarily unde two; those that undergo two do not nee sarily undergo three; and so forth. Not that divide grow to be countable coloni

In figure 5, where you compared growth rates of the irradiated with the irradiated ones, there is a slight falloff the case of the irradiated cells. The ger ation times of the controls and irradia cells was about 11 hours. From about to 100 hours, however, the irradiated c appear to be lagging (i.e., growing rapidly) and in this region they have dergone 5 to 6 fissions.

Elkind: That's right.

Powers: We have noted before in P *amecium* that there might be someth magic about 6 cell divisions, that the c may very well be running out of thi after 6 divisions. So, the fact that a divides several times does not mean tha will produce a clone. The question h is: What is the successfully surviving in your material?

Elkind: What we did in this exp ment was to count cells without fixing staining with phase microscopy. In the gion from 60 to 100 hours, the clones c tained an average of about 30 to 150 c per clone. I think it is perfectly reasona that we might have had a 10 to 20% er and just couldn't count all the cells curately enough in this region since th cells form tightly packed clones.

In addition, we have done grov curves, both for the controls and for irradiated population, by the method trypsinizing as a function of time. have observed exponential growth for t decades at least, after which in both stances we begin to see a drop-off wh may be due to a number of reasons wh are beside the point in question.

There is another factor. We also ha evidence that not all the progeny of initially surviving cell either have the ca bilities of growing at the same avera initial rate, or of undergoing the sa number of divisions. Some of these pr eny lines are "dead-end" lines. I think t is one reason why, when large doses of ra n are used, there is a distribution in ony sizes after a given length of postir-iation incubation. That is, dead-end geny lines could very easily contribute uch a distribution.

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[5] L. Revesz, Stanford University.

[6] E. L. Powers, Argonne National Laborato

ACOBSON[7] : I would like to make some nparisons between this data and some the data on larger micro-organisms, ticularly on *Chlamydomonas reinhardi.* n Chlamydomonas, there is a recovery shown by dose fractionation, with most he recovery occurring within less than f an hour. But by the criterion of re-nption of cell division, it takes nearly a before the irradiated cells resume their division.

My belief is that these are two different es of recovery. Also, I have a statement to what is killing the cells. This organ-has a rather complicated division cycle olving successive endomitotic division. appears that, generally speaking, the s always die at the same point in the ision cycle—that is, in the uninucleate tes—as though there is some critical nt in the postirradiation life cycle of the which they may or may not get ough, even though they have gone ough a normal cell division cycle after diation, before death.

KAPLAN[8] : These observations are all sed on cells with very short generation es *in vitro.* In short, they are growing out as fast as mammalian cells are able grow. Clearly, any interpretation of h results in terms of *in vivo* systems uld have to take into consideration the t that very few cells in mammals *in vivo* w at this rate.

Does Dr. Elkind have any observations cells *in vitro* which have deliberately en grown at lower temperatures or under nditions which would force them to grow th a longer generation time, and whether der these conditions some of these pa-meters change?

ELKIND: I can't answer that question mpletely. First of all, we have looked at covery at room temperature in Chinese mster cells. It does occur, although at slower rate, and even though these cells not grow at room temperature. Second, . Lockart in our laboratory has done a nited number of studies on recovery with La S-3 cells using three different sublines, all of which had generation times about twice that of the Chinese hamster cells. The recovery kinetics appear to be similar. And third, I think that your initial remarks contained an assumption; namely, that there ought to be some relationship between rate of growth and the presence or absence of recovery. This may be true, but in my view, until it has been shown to be true we have no basis to assume it is true.

KELLY[9] : Dr. Kaplan's remarks introduce what I want to say. I will attempt to answer his comment on the question of whether tissue cultures are the proper model for mammalian radiation survival. If you recall Dr. Elkind's survival curve, at 600 r roughly 1% of the cells are capable of forming a macro colony, in other words, capable of continual division. In mice the LD_{50} region is of the order of 600 r. We also know that it is necessary to inject of the order of 1% of the bone marrow cells normally present, in order to have a mouse survive a lethal radiation dose. In other words, if 600 r allows 1% of the bone marrow stem cells to survive, the mammalian data fit very well with tissue culture data.

Dr. Elkind's recent experiments show rapid recovery of irradiated cells which have not suffered a sufficient number of hits to be killed. A large body of mammalian survival data has accumulated in which the radiation was either fractionated or given over a protracted period of time. In these experiments a rapid recovery component has usually been found. For example, if the dose is protracted over a 24-hour period the LD_{50} for X rays is approximately twice as great as the LD_{50} for a radiation exposure lasting only a few minutes. Dr. Elkind's data now give a cellular basis for these observations. If the dose is protracted, there is time for recovery in the bone marrow stem cells so that it takes twice the dose to get down to the 1% survival level.

I think this ties together many experiments in the mammalian literature which have been very puzzling.

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[7] B. S. Jacobson, University of Texas, Austin.

[8] H. S. Kaplan, Stanford University.

[9] L. S. Kelly, University of California, Berkeley.

ELKIND: Although I don't think that our limited studies with cell cultures should be underestimated, I don't think they ought to be over interpreted either. Dr. Kelly referred to a dose-rate effect in animals which might be consistent with the recovery observations we have made after acute exposures. This suggests that similar dose-rate effects might be observed with cultured cells. But there are also suggestions from mammalian experiments that recovery processes may not take place while the animal is being exposed. In any event, dose rate measurements with cells have not been made and I prefer not to undertake models to explain animal effects unless I have a cellular basis to start with.

ALEXANDER[10]: In connection with Dr. Kaplan's comment, we have many instances using mouse lymphoma cells in tissue culture to show that radiosensitivity depends upon the physiological state of the cell and in particular on its nutritional status. The radiation response of slow growing or almost stationary cultures is very different indeed from that of cells in an ideal medium where they divide every 10–11 hours. In the rapidly dividing cells we have observed recovery with fractionated doses of exactly the same type as seen by Dr. Elkind. However, recovery is much less marked if the cells are irradiated under conditions of restricted division (e.g., when folic acid is withdrawn). Consequently one can't argue about the value or otherwise of dose fractionation in radiotherapy from tissue culture experiments. They probably give us a guide to *in vivo* behavior of bone marrow but not of tumors whose cells do not divide once a day or more.

ELKIND: First, was your survival higher for a single dose for the slower growing cells? And second, was there no particular evidence of an increased survival with fractionation?

ALEXANDER: That is right. The experiment is as follows: (a) lymphoma cells maintained in complete tissue culture medium. Division time 10 hours (b) lymphoma cells kept in incomplete medium (division time 18 hours) and transferred immediately before irradiation to the complete medium. Both systems show an equal
survival of approximately 10% for a si
dose of 300 r. The dose is fractionated i
two lots of 150 r separated by 4 to 6 hou
Then the survival of (a) is about 5
while that of (b) is about 15%, i.e., mu
less recovery in the case b. There is
other difference between these two cultu
and that is that in (a) with doses up
500 r all cells divide at least once bef
dying (i.e., autolysing at about three t
days after irradiation). While in (b)
cells do not in general divide before dis
pearing. I interpret this as meaning t
cell division or attempts thereat are
necessary for these cells to die and t
consequently chromosomal damage is
the important cause of death for *th*
cells. I should point out that our surviv
were determined not by cloning but by
trapolation from the growth curve 6 t
days after irradiation. This method
calculation is liable to serious error if th
is a significant mitotic arrest. We do
believe that there is a serious mitotic arr
for the reasons summarized in P. Al
ander and Z. B. Mikulski (Bioche
Pharm., *5*: 275, '61).

ELKIND: Dr. Alexander's point be
also on some experiments already in
literature. We don't know whether the s
vival curve for his cells is the same as ou
but let me assume it is similar.

At a dose of about 400 or 500 r, we
down to about 0.2 survival in our ca
That is about the beginning of the
ponential region. If the first dose
smaller, complete recovery would be rep
sented by a curve similar in shape to
initial one but displaced only by a sm
amount to the right (higher doses).
cells which are given large enough doses
reduce the survival to the exponential
gion, complete recovery would be rep
sented by a curve shifted a larger distan
to the right.

On our scale (which may or may not
a valid comparison) 300 r would be so
where around 50% survival and the eff
of recovery would be small. I am i
pressed with the fact that Alexander
ready sees some recovery even at this d
for the 10 hour cells. If, then, for the
hour case, the survival to 300 r was larg

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[10] P. Alexander, Chester Beatty Research In
tute.

would have been even more difficult to olve the influence of recovery.

There are two additional reasons why Alexander's results with respect to the estion of recovery versus growth rate y be misleading. First, in his experi- nt, clonal formation is not the criterion survival. Dr. Alexander infers what the t survival is by extrapolating the postir- diation growth curve back to zero time. he has pointed out, this method is valid ly if there is no division delay induced the irradiation. Since there most cer- nly must have been division delays ich must also have increased with dose, d since it is quite likely that the delay a given dose was larger for the cells ich came from a deficient medium, the sis for comparing the slow and rapidly owing cells becomes tenuous. Further, timating survival by the extrapolation ethod always leads to an underestima- n of the survival. If for a given dose the wer growing cells had a longer delay, ey would appear to have had a lower sur- val while they might in fact have had an ual or greater survival than the rapidly owing cells.

The second reason concerns the kinetics recovery. Four to 6 hours in our ex- riments is in the region in which the t survival is changing rapidly. It is quite asonable that the net survival to the two posures might have been different only cause the cardinal points on their re- ective recovery curves were displaced lative to each other.

Powers: Do you require that all the rvivors which you score go through the me number of cell divisions?

Elkind: In a rough way, yes. They ust all exceed a limited number of divi- ons.

Powers: Does this go through three cell visions, 5 cell divisions, or 15?

Elkind: At the end of about 13 days of owth any clones that are smaller than out 200 cells are not acceptable as repre- nting an initially viable cell. Our largest ones at that time may have 10^5 cells.

Powers: But you do then have from 00 to 10^5 cells in the clones included in e survival curves?

Elkind: We have a spectrum of clone zes after large doses.

Kelly: Dr. Alexander's comment can be turned around to say that cells which are unhappy and therefore have slow generation times, behave unpredictably and not like normal cells.

Kaplan: There is need for extreme caution in the extrapolation of these admittedly very beautiful and precise data, to the very imprecise situations which one encounters in clinical practice.

To the best of my knowledge, there are virtually no tumors in patients that have generation times comparable to those *in vitro*. Moreover, the generation times in different cells within a tumor are vastly different. For this reason, I think that those who may read this work and who may be tempted to leap to the conclusion that fractionation has no proper place in radiotherapy should be warned to be careful.

Celada[11]: I would like to tell Dr. Kaplan that in our laboratory Dr. Capalbo has shown that also antibody-forming cells in the presence of antigen have a maximum generation time of 12 hours.

Kelly: I think in the literature you will find that essentially all the "stem cells" in the mouse have generation times of the order of 12 to 24 hours. This holds for lymph nodes, bone marrow, small intestine, essentially all the generative cells that have been measured to date.

Whether this applies to the dividing tumor cells, I don't know and I don't think anyone else knows at the moment. The difficulty is that in a tumor, there is no marker for the generative cells. Until experiments are done in which thymidine is infused over a long period of time under conditions where the mouse and the tumor remain healthy, we don't know whether in a growing tumor there are small groups of cells which have rapid generation times or whether each cell divides more rarely than do the normal generative cells.

Mendelsohn[12]: In answer to Dr. Kelly, I have some as yet unpublished data which may supply the information she has called for. Tritiated thymidine has been infused for as long as 7 days into C3H mice carrying primary breast tumors. Without going into details, I can say that analysis of autoradiographs of such tumors after infusion has indicated that a sizable fraction of tumor cells remain unlabeled. In addition, a small but detectable number of the unlabeled cells can be found in mitosis 4 days after the end of the infusion. I interpret this data to mean that both the above complications exist in these tumors. As Dr. Kelly implied, a major constituent of these tumors does not contribute to tumor cell proliferation; and as Dr. Kaplan has indicated, the growing tumor cells probably have widely divergent growth rates.

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[11] Franco Celada, Oak Ridge National Laboratory.

[12] M. L. Mendelsohn, University of Pennsylvania.

Upton[13] : Dr. Oakberg may well take me to task, but my impression is that the generation time for spermatogonia is probably substantially longer than that in other germinal cells of the tissues you mentioned.

It also is my impression that in this system there is less recovery after a single dose of radiation, suggesting in fact that there may be some correlation between generation time and rate of repair.

On the other hand, there is the paradox that in the mouse oocyte we have evidence of intensity dependence; yet this is not supposed to be a mitotically active cell. (See Oakberg in this volume.)

Revesz: In an attempt to extrapolate results obtained in *in vitro* and *in vivo* situations if might be of interest to conduct studies similar to those described by Dr. Elkind also under anoxic conditions.
mor cell populations are to a great ext
anoxic *in vivo*. For such population
conclusions drawn from *in vitro* exp
ments performed with well oxygena
cells might not be relevant.

Elkind: This is something we h
started to work on.

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[13] A. C. Upton, Oak Ridge National Laborat

odification of Mutagenesis Initiated by Ultraviolet ght through Posttreatment of Bacteria th Basic Dyes[1,2,3]

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Department of Medicine, State University of New York, Downstate Medical Center, Brooklyn, New York

o induce a mutation, a mutagenic
nt must somehow provoke a stable
nge in the sequence of nucleotide pairs
ed together in the molecules of genic
xyribonucleic acid (DNA). When auxo-
phic bacteria are exposed to ultraviolet
t (UV), mutations to prototrophy are
uced, but these alterations of the ge-
ic code are rarely, if ever, obtained as
ect and immediate consequences of the
orption of photons. The fate of the
ential prototrophs remains uncertain
about an hour after irradiation. Con-
ons during this "sensitive period" de-
mine whether they will be "fixed" as
ble mutants or lost irrevocably. This
ile phase of the induction process, when
ttreatments can affect the ultimate
ld of induced mutations, is ended by the
urrence of a decisive event that stabi-
s the genotype of the potential proto-
ph.

ecent investigations of UV-induced
totrophy have shown that the course of
tagenesis depends upon the pattern of
thetic activity during the early part of
first postirradiation cell division. Po-
tial prototrophs disappear rapidly and
eversibly when conditions after irradia-
n are unfavorable for the synthesis of
teins and ribonucleic acid (RNA) as in
nedium devoid of amino acids or in one
taining chloramphenicol (Witkin, '56;
udney and Haas, '58) or 6-azauracil
oudney and Haas, '59b). After less than
hour under such "low-frequency" con-
ions, the yield of mutations can no
ger be raised by the addition of amino
ds, nor by the withdrawal of the in-
itor. The number of potential proto-
phs fixed in stable mutant form is much
ger if conditions during the sensitive period favor the synthesis of RNA and proteins, as in a medium rich in amino acids (Witkin, '56). After an hour under such "high-frequency" conditions, the yield of mutations can no longer be reduced by the addition of chloramphenicol, nor by amino acid deprivation. Potential mutants are maintained in the "promutant" state for relatively long periods of time when synthetic activity in general is suspended or inhibited after irradiation, as in saline solution or medium without a carbon source (Witkin, '58), or by the presence of dinitrophenol (Doudney and Haas, '58). Finally, the sensitive period is, itself, subject to the influence of posttreatments: mutations are lost or fixed more slowly when cell division is retarded by caffeine, or by increasing doses of UV light (Witkin, '58), and fixation is also delayed by thymine deprivation in a thymineless strain (Weatherwax and Landman, '60). These observations suggest that the stabilization of the genotype of potential prototrophs, whether by fixation or by loss, is intimately associated with the behavior of DNA. Fixation of mutations has been linked to DNA synthesis by Lieb ('60) and by Weatherwax and Landman ('60), but Doudney and Haas ('59a, '60) considered the irreversible loss of potential prototrophs to result from the disappearance of modified RNA precursors produced by UV light, through processes independent of DNA synthesis. If the loss of potential mutations under

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[1] These investigations were supported in part by research grant E-1240 from the National Institutes of Health.

[2] This paper is dedicated to Professor L. C. Dunn in recognition of his long and distinguished career.

[3] This work was carried out with the efficient assistance of Mr. Nicholas Sicurella.

low-frequency conditions, as well as their fixation under high-frequency conditions can be shown to depend upon the behavior of DNA, the idea that mutagenic intermediates are involved in the induction process will become difficult to support. Such a finding would be more compatible with the idea that UV light initiates mutagenesis by producing unstable modifications directly in genic DNA.

The experiments to be described, in which basic dyes were used as postirradiation metabolic inhibitors, were designed primarily to determine whether the same posttreatments that delay or prevent the loss of potential mutations are also capable of interfering with their fixation.

MATERIALS AND METHODS

A tyrosine-requiring derivative of *Escherichia coli* B/r, designated as WU36, was used. Cultures were grown overnight, in Difco Nutrient Broth, with the addition of 0.5% NaCl, on a reciprocal shaker at 37°C. Ten milliliters of culture were added to 90 ml of fresh nutrient broth, incubated for 40 minutes with shaking, then chilled for 10 minutes at 5°C. This suspension of bacteria in the late lag phase was then centrifuged; the pellet was rinsed and resuspended in saline solution to give a titer of about 10^9 bacteria per ml.

Irradiation was carried out by exposing 8 ml of the saline suspension of bacteria in an open Petri dish, with constant agitation, to 400 ergs per mm^2 of UV light, using a General Electric Germicidal lamp. Subsequent operations were illuminated by yellow light to avoid photoreactivation.

Posttreatments were administered by diluting 1 ml aliquots of irradiated bacterial suspension with 9 ml of the desired liquid in test tubes, and incubating at 37°C. for the desired time. Posttreatments were terminated by plating out to determine survival and mutation frequency.

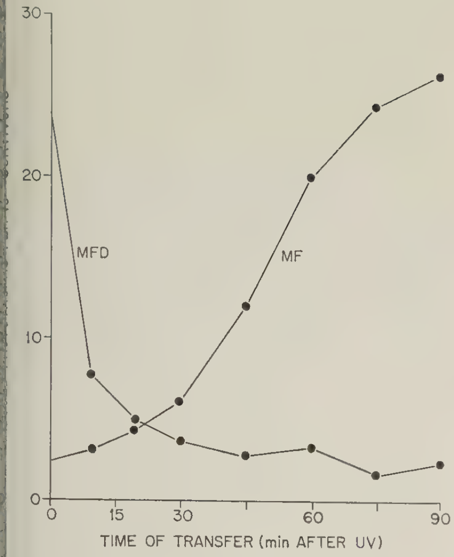
Transfers from one solid medium to another were made by washing bacteria from the surface of the first plate with 3 ml of saline solution, and replating aliquots of the wash.

The solid medium used for both survival and mutation determinations was a minimal glucose-salts medium—the medium "E" of Vogel and Bonner ('56)—supplemented with 5% liquid nutrient br
This semienriched minimal agar (
SEM) contains enough tyrosine to per
ty$^-$ cells to form small but visible colo
when dilute suspensions are plated for
vival assay, and to allow a massive in
ulum of about 10^7 cells to undergo sev
cell divisions on the plate, thus permitt
the phenotypic expression of any ty$^+$
tants present. Prototrophic colonies g
to a large size against the thin film of
ited parental growth. Other amino a
are present in amounts sufficient to per
maximum fixation of mutations. Plat
out on 5% SEM agar, therefore, provi
high-frequency conditions, and failure
prototrophs to appear means that irrev
ible loss has preceded plating.

Pyronin B, crystal violet, methylene bl
and toluidine blue were obtained from
National Aniline Division of the All
Chemical and Dye Corporation, neu
acriflavine and sodium deoxyribonucle
from the Nutritional Biochemicals Cor
ration, and methyl green from Hartm
Leddon Company; chloramphenicol
generously supplied by Charles Pfizer
Company.

EXPERIMENTAL RESULTS

We have developed the view that
rate at which potential mutants are l
as well as the rate at which they are fix
measures the occurrence of a stabiliz
event associated with the behavior of D
The rates of fixation and loss may be c
veniently determined in the kinds of exp
iments illustrated in figure 1. To meas
the irreversible loss of potential mutatio
the irradiated bacteria are maintained
itially under low-frequency conditions
this case, on minimal agar supplemen
only with a small amount of tyrosine), a
are transferred, after varying periods
time, to high-frequency conditions (5
SEM). As mutations are irreversibly l
the transfer becomes increasingly ineff
tive in raising the yield of mutations, wh
is stabilized completely, at the low le
characteristic of the initial condition, af
about 30 minutes. To measure the i
versible fixation of mutations, the pro
dure followed is essentially the reverse,
initial incubation under high-freque
conditions being followed by transfer, af



g. 1 Mutation frequency decline and muta-
fixation. Aliquots of irradiated suspension
ad on surface of 5% SEM agar and minimal
supplemented with 1 μg tyrosine per ml;
es incubated at 37°C., transfers made after
ous times. For MFD, transfer was from mini-
tyrosine agar to 5% SEM agar. For MF,
sfer was from 5% SEM agar to minimal-
sine agar.

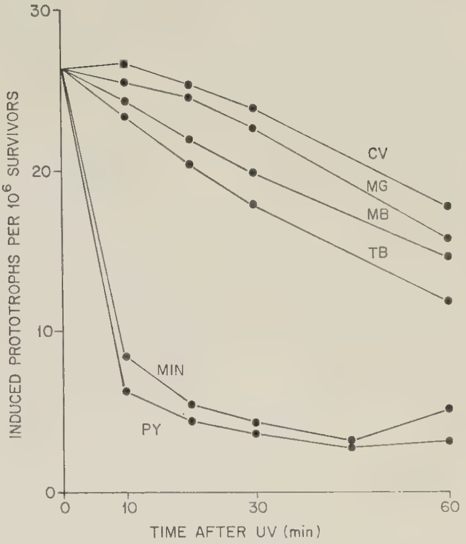


Fig. 2 Effects of posttreatment with basic dyes on mutation frequency decline. One-milliliter aliquots of irradiated suspension added to 9 ml minimal medium supplemented with 1 μg tyrosine per ml, with or without the addition of basic dyes in final concentrations as follows: MG (methyl green), 0.01%; CV (crystal violet), 0.0001%; Py (pyronin), 0.0005%; MB (methylene blue), 0.001%; TB (toluidine blue), 0.001%. Samples were plated out on 5% SEM after various times. Survival was not significantly affected.

ied time intervals, to low-frequency
r. The "challenge," in this case with
no acid deprivation, entirely loses its
ver to reduce the yield of mutations
r about 60 minutes on the amino acid-
iched medium. Doudney and Haas
3) have designated these processes
tation frequency decline," or MFD, and
tation fixation," or MF, and in this dis-
sion we shall adopt these designations
convenience.

asic dyes, used in these experiments as
ttreatments, were tested for possible
cts on MFD and MF.

Effects of basic dyes on MFD

igure 2 shows the effects of 5 basic
s on the loss of potential prototrophs
der conditions of amino acid depriva-
n. Irradiated bacteria were incubated in
nimal medium supplemented only with
g of tyrosine per ml, with periodic plat-
out on 5% SEM agar. The usual MFD
ve is obtained, with the yield of muta-
ns fully stabilized at a low level after
ut 30 minutes. Pyronin fails to affect the course of MFD, which is markedly slowed, however, by methyl green, crystal violet, methylene blue, and toluidine blue. All of the dyes were used in concentrations that prolonged the time required to complete the first postirradiation cell division by about one hour. Pyronin, which is unique among the basic dyes in the selective affinity that it exhibits under certain conditions for RNA (Kurnick, '55), not only fails to retard MFD, but is capable of causing MFD when applied under otherwise high-frequency conditions, as in nutrient broth. Pyronin thus duplicates in all essentials the action of chloramphenicol, amino acid deprivation, and 6-azauracil. All the other dyes are known to stain DNA-containing structures, methyl green exhibiting a high degree of specificity in its affinity for polymerized DNA (Kurnick, '55).

Figure 3 shows the action of acriflavine, the most efficient of all the dyes tested in blocking MFD. The experiments with acriflavine are of particular interest since the

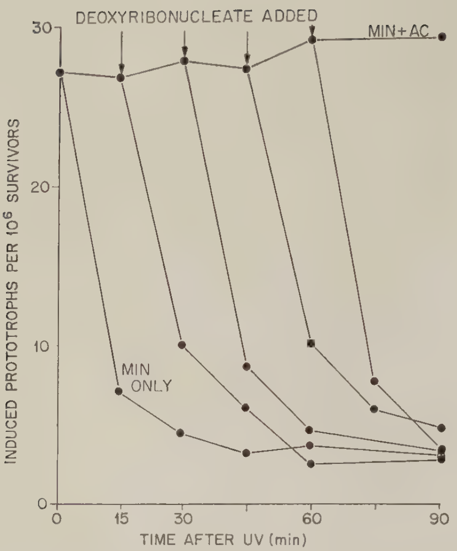


Fig. 3 Release of acriflavine-blocked MFD by sodium deoxyribonucleate. One-milliliter aliquots of irradiated suspension added to 9 ml minimal medium supplemented with 1 μg tyrosine per ml, with or without the addition of acriflavine (final concentration 0.0001%). At times indicated by arrows, sodium deoxyribonucleate was added to one of the acriflavine-containing tubes (final concentration 0.5%). Samples were plated out on 5% SEM agar after various times. Survival was not significantly affected.

biological effects of this compound are completely reversed by the addition of an excess of sodium ribonucleate (McIlwain, '41) or deoxyribonucleate. In figure 3, the arrows indicate the times when deoxyribonucleate was added to the acriflavine-blocked MFD tubes. It will be seen that removal of this dye from combination with cellular components permits the immediate onset of MFD. Acriflavine is known to combine with DNA (Lerman, '61).

Effects of basic dyes on MF

Figure 4 shows that all the basic dyes that retard the loss of potential mutants under low-frequency conditions also delay the fixation of potential mutants under high-frequency conditions. Irradiated ty^- cells were incubated on 5% SEM plates with or without the addition of division-retarding concentrations of dyes, and "challenged" by transfer to minimal agar supplemented only with 1 μg of tyrosine per ml. Similar results were obtained when

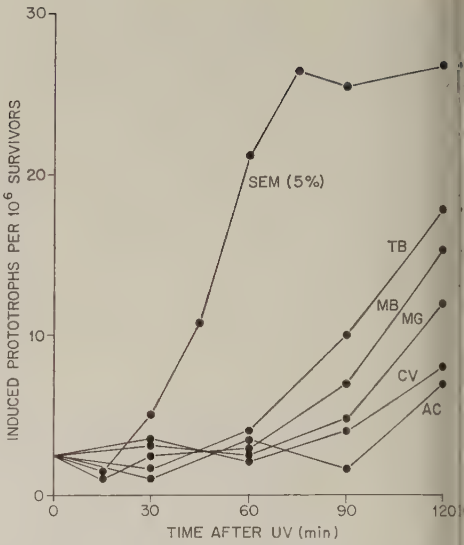


Fig. 4 Effect of basic dyes on mutation fi
tion. Aliquots of irradiated suspension spread
surface of 5% SEM agar with or without the ad
tion of basic dyes in final concentrations as
lows: Ac (Acriflavine), 0.0001%; MG (met
green), 0.002%; CV (crystal violet), 0.0001
MB (methylene blue), 0.02%, and TB (toluid
blue), 0.001%. After various times, trans
were made by plate-washing and replating
minimal agar supplemented with 1 μg tyros
per ml. Survivals remained constant, except
sampling error, as determined by assays at
time of transfer.

the fixation of mutants was challeng
with a 60-minute incubation with chl
amphenicol or pyronin.

Enhancement of the mutagenic potency ultraviolet light by basic dyes that delay MFD and MF

Caffeine, which delays both MFD a
MF, was found to cause a marked increa
in the yield of UV-induced prototrop
when added to 5% SEM agar, althou
it showed no mutagenic activity of its o
in unirradiated controls (Witkin, '58). T
enhancement was most effective at l
doses that are ordinarily followed by
tremely short sensitive periods. The mu
tion-dose curve obtained on caffeine-su
plemented plates approached linearity,
contrast to the steeply exponential rise
mutations with dose ordinarily observ
This result was interpreted as evider
that caffeine permits the fixation of
larger fraction of the potential mutants

iderable portion of which appear to be
even under high-frequency conditions.
exponential mutation-dose curve ordi-
y observed for induced prototrophs
interpreted as evidence for "self-en-
ement" by increasing doses of UV
that prolong the sensitive period in-
singly in the low-dose range. The pre-
on was made that any posttreatment
ble of extending the sensitive period,
relatively little inhibition of the syn-
s of proteins, would increase the yield
ototrophs above the usual "maximum"
ined under high-frequency conditions.
flavine, methyl green, crystal violet,
dine blue, and methylene blue, all of
h delay MFD and MF, as well as
nin, which does not, were added to
SEM agar in concentrations that per-
cell division at a reduced rate. Table
ows the results of plating both un-
liated and irradiated bacteria on these
supplemented high-frequency plates.
radiated bacteria incubated on the
containing plates give rise to proto-
hs no more frequently than controls
ving on 5% SEM agar without added
, demonstrating the nonmutagenicity
he dyes in this material. Bacteria ir-
ated with low doses of UV light, how-
, show the reduced yield expected in
presence of pyronin and varying de-
s of enhancement, up to more than
tenfold, when incubated on plates containing any of the other dyes. It is clear that all of the dyes capable of delaying the occurrence of both MFD and MF are also found to raise the yield of mutations above the usual maximum when present under high-frequency conditions. Acriflavine, the most effective dye in retarding MFD and MF, was also found to produce the highest degree of enhancement.

Figure 5 shows the frequency of prototrophs obtained after exposure to two low doses of UV light by plating on a variety of postirradiation media. Variations in survival are negligible at these doses as compared to the large differences in mutation frequency. At the low-frequency end of the spectrum, the yield may be raised by increasing the amount of amino acid enrichment, or by removing inhibitors of RNA or protein synthesis. The rate of growth is maximal at an enrichment level of about 5%, and the mutation frequency cannot be raised further by additional nutrient broth or amino acid enrichment. The yield of mutations can be further increased, however, by the addition of inhibitors that delay MFD and MF. Considering the continuum of mutation frequencies obtainable under these conditions, there is no reason to suppose that high-frequency conditions necessarily permit the fixation of 100% of the potential proto-

TABLE 1

Effects of addition of basic dyes to the postirradiation culture medium on the frequency of ultraviolet-induced prototrophs

Final concentrations of dyes added to 5% SEM agar: pyronin (Py), 0.0005%; methyl green (MG), 0.002%; crystal violet (CV), 0.0001%; methylene blue (MB), 0.02%; toluidine blue (TB), 0.001%; acriflavine (AC), 0.0001%. Plating volume 0.1 ml in all cases.

UV dose	Added to agar	Ty^- colonies (10^{-6} dilution)	Ty^+ colonies (10^0 dilution)
0	—	175, 204	9, 6
	Py	163, 188	14, 12
	MG	210, 202	4, 7
	CV	160, 169	5, 11
	MB	181, 188	5, 5
	TB	171, 190	13, 8
	Ac	169, 208	5, 5
100 ergs/mm^2	—	183, 195	237, 256
	Py	149, 160	42, 31
	MG	168, 187	1560, 1380
	CV	118, 123	1260, 910
	MB	175, 163	841, 896
	TB	181, 203	736, 812
	Ac	149, 160	2900, 3960

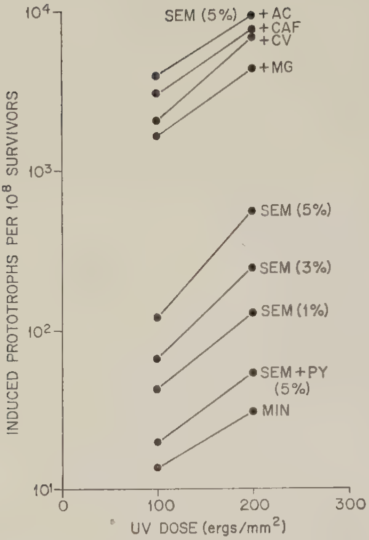


Fig. 5 Frequency of induced prototrophs obtained on various postirradiation plating media. Aliquots of irradiated suspension plated on minimal medium supplemented only with 1 μg tyrosine per ml (Min), on 5% SEM containing 0.0005% pyronin (Py); 0.0001% acriflavine (Ac); 0.1% caffeine (Caf); 0.002% methyl green (MG); 0.0001% crystal violet (CV); 1% SEM; 3% SEM, and 5% SEM. Survival range for 100-erg dose, 94–100%; for 200-erg dose, 74–91%.

trophs. The nonmutagenicity of these dyes in unirradiated bacteria suggests that the enhancement represents fixation of a larger fraction of the UV-induced promutants, rather than the additive or synergistic effect of independent mutagens.

DISCUSSION

The finding that basic dyes capable of interfering with mutation fixation are also effective in blocking mutation frequency decline makes it necessary to consider the possible relation of MFD to the behavior of DNA. There seems little doubt that the fixation of mutations is intimately associated with DNA replication. This conclusion is based on the finding that MF is blocked by thymine deprivation in a thymineless strain (Weatherwax and Landman, '60), and that fixation as determined by a photo-reversal challenge also parallels the synthesis of DNA (Doudney and Haas, '60). The metabolic inhibitors found to retard MF, including UV light and caffeine (Witkin, '58), acriflavine, methyl green,
crystal violet, methylene blue, and to
dine blue, have, as a common denomina
the ability to inhibit DNA synthesis,
at least two (UV light and methyl gre
are highly specific in their effects on D
The association of MF with DNA synth
thus appears to be established bey
serious doubt.

Accepting the relation between MF
DNA replication, what significance can
ascribe to the blockage of MFD by
same metabolic inhibitors that block fi
tion? First of all, it is clear that MFD be
no direct relation to the net synthesis
DNA. MFD runs far ahead of DNA s
thesis in time (Doudney and Haas, '59
and its occurrence to completion does
substantially alter the subsequent rates
RNA, protein, or DNA synthesis (Doud
and Haas, '60). Moreover, MFD is
blocked by thymine deprivation in a t
mineless strain (Weatherwax and La
man, '60). The absence of any correlat
between MFD and net synthesis of DN
further underlined by the finding of Har
and Ziporin ('58) and of Draculić
Errera ('59) that UV-irradiated bacte
are unable to recover the ability to s
thesize DNA if protein synthesis is inh
ited (by chloramphenicol or deprivation
a required amino acid) immediately a
the exposure. Thus, although MFD is
tarded or prevented by many inhibitors
DNA synthesis, some quite specific in th
effects on DNA, its course bears no re
tion to measurable increase in DNA.

These observations may be most read
accounted for by the assumption that
primary effect of UV light in initiat
mutagenesis is the production of unsta
changes directly in genic DNA. Doud
and Haas ('58, '59a, '60) have propose
hypothesis presuming an indirect mec
nism of DNA replication, according
which UV-modified RNA precursors tra
fer new information to subseque
formed DNA through an RNA-protein
termediate. Lieb ('60) invokes an unsta
mutagen, decaying at a rate indepe
ent of DNA synthesis, the concentrat
of which at the time of DNA dupli
tion determines the yield of mutatio
According to any hypothesis assuming t
MFD measures the loss of mutagenic in
mediates, or of mutagens decaying a

independent of DNA synthesis, it
ld be difficult to account for the block-
of MFD by inhibitors that also block
and DNA replication.

arting with the assumption that the
ary target of UV light is DNA itself,
pothesis can be developed to account
he following major observations: (1)
potential mutations are lost under
litions unfavorable to protein or RNA
hesis; (2) that potential mutations are
l under conditions favorable to protein
RNA synthesis; (3) that many inhib-
s of DNA synthesis retard or block both
D and MF; (4) that thymine depriva-
in a thymineless strain blocks MF, but
MFD, and (5) that inhibitors capable
nterfering with MFD enhance the mu-
nic potency of low doses of ultraviolet
t.

ssuming that mutagenesis begins with
production of reparable damage in
A, the loss of potential mutations under
frequency conditions may be consid-
l to result from repair of the UV-pro-
ed damage. The presence of amino
s under high-frequency conditions
t be assumed to interfere with repair,
haps by some physical association of
machinery of RNA and protein syn-
sis with DNA. MFD, therefore, is re-
before replication; MF is replication
hout repair (or with imperfect repair).
inhibitors that interfere with both
D and MF might do so through a single
ct on DNA, altering its properties in
h a way as to prevent both repair and
lication. Such a dual action would
easily imagined in the case of acri-
ine, for example. Acridines are known
combine with the DNA backbone, prob-
y by intercalation between the bases,
h extension of the backbone and revers-
destruction of the helical properties of
molecule (Lerman, '61). Such modifi-
ion of the DNA helix might well inter-
with both repair and replication. Thy-
ne deprivation in a thymineless strain,
vever, would be expected to interfere
y with the production of new DNA, and
uld therefore prevent MF, but there
ld be no good reason to expect any
rference with repair in this case. The
ling that thymine deprivation blocks MF but not MFD (Weatherwax and Landman, '60) confirms this view.

According to this DNA-repair hypothesis, the enhancement obtained by posttreatment of irradiated auxotrophs with MFD-retarding agents is readily explainable as another manifestation of their action in preventing repair. If we assume that, at low doses of UV light, there is considerable repair even under high-frequency conditions, so that a large fraction of potential prototrophs is ordinarily lost, the action of caffeine, acriflavine, and the other "enhancers" may be interpreted most simply as the "rescue" of these promutants through prevention of repair. In other words, the "enhancers" stabilize the UV-provoked lesions in much the same way as do amino acids, but they do so more efficiently than amino acids at low doses of UV. If enhancement is indeed the consequence of repair-prevention, as proposed here, thymine deprivation in a thymineless strain, which blocks MF (replication) but not MFD (repair), should cause no enhancement. No enhancement has been described as a consequence of thymine starvation (Weatherwax and Landman, '60).

Further evidence that the primary target of UV light in the induction of prototrophs is DNA comes from the photoreversibility of the potential mutations (Doudney and Haas, '60). Potential prototrophs are eliminated (before DNA replication) just as effectively by exposure to visible light as by incubation with chloramphenicol, strongly suggesting that the same premutational lesions are being affected by the two kinds of posttreatment. Recent work on photoreactivation has shown that enzymatic repair of damage to DNA is the basis of photoreversal of effects produced by UV light (Goodgal *et al.*, '57; Rupert *et al.*, '58; Rupert, '60). The loss of mutations under conditions of amino acid deprivation or chloramphenicol inhibition, therefore, may well reflect the operation of an alternative mechanism of repair, operating in the dark under low-frequency conditions, of the same initial damage.

SUMMARY

Posttreatment with basic dyes, in concentrations that retard cell division, was found to influence the induction of muta-

tions to prototrophy by UV light in a tyrosine-requiring strain of *E. coli*. Pyronin, which is unique among the dyes in its selective affinity for RNA, was found to duplicate the effects of chloramphenicol or amino acid deprivation in causing the rapid and irreversible loss of potential prototrophs (mutation frequency decline, or MFD). Acriflavine, methyl green, crystal violet, methylene blue, and toluidine blue, all of which are known to combine with DNA, delay or retard the occurrence of MFD under conditions of amino acid deprivation. When acriflavine is removed from its combination with cellular components by the addition of an excess of sodium deoxyribonucleate, MFD begins promptly. The same basic dyes that delay MFD were also found to interfere with the fixation of mutations (MF) in an amino acid - enriched medium, and to cause marked enhancement of the mutagenic potency of low doses of UV light. While showing no independent mutagenic activity for unirradiated bacteria, all the dyes except pyronin increased the yield of induced mutations significantly when added to the enriched medium upon which irradiated bacteria were incubated.

These results were interpreted as evidence that UV light initiates mutagenesis by producing unstable changes directly in genic DNA. MFD is interpreted as a repair process, blocked by the machinery of RNA and protein synthesis and by the presence of certain basic dyes.

OPEN DISCUSSION

LIEB[4]: I would like to say that I never did say, or mean to say, that I thought the primary product was not in the DNA. I want to ask Dr. Witkin how she thinks UV produces a non-complementary base in the DNA.

WITKIN: Several ways have been proposed that make it feasible to suggest this as a possible effect of UV. The hydration of cytosine, for example, could lead to this result.

LIEB: The only solid hypothesis I have heard at this meeting is the formation of the thymine dimer. Will a thymine dimer fit the hypothesis?

WITKIN: My hypothesis does not propose any specific kind of damage. It suggests that the primary lesion is in D and that the loss of mutations is du repair. It is quite possible to think of i terms of dimer formation, although ot kinds of damage would fit equally w The data I have presented do not bear the specific nature of the UV-indu damage.

AUERBACH[5]: I wish to make a st ment about something which I thou was misleading. It is the statement t posttreatment protein synthesis is not n essary after mutagenic chemical agen I discussed this with Strauss. It may his interpretation is right, but I think evidence is not convincing.

Strauss induced tryptophan reversi in *E. coli* either by UV or by di-ethylsulf Both treatments produced more mutati when plating was done on broth than wh it was done on minimal medium. The o difference was that when he kept treated bacteria for one hour in liq medium without protein synthesis then plated on broth, the number of mu tions induced by UV was drastically duced, while the number of chemic induced mutations was the same as wh he plated directly on broth. I think all can conclude from this experiment is t one hour of postincubation without p tein synthesis is too short a time to red mutation frequency after the chemi treatment. In fact, when the posttre ment was followed by plating on minin medium, there was the same effect of posttreatment after chemical treatment after UV. Dr. Strauss has a different int pretation for this, but without further e dence mine seems the simpler.

KIMBALL[6]: I would like to raise a po of interpretation. In experiments w mutation frequency decline, there usually two periods to consider, the per of incubation in some agent or medi that "promotes" decline and the sub quent period after plating and before final fixation of mutation by DNA s thesis. Only the duration of the form

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[4] Margaret Lieb, University of Southern C fornia, Los Angeles.

[5] Charlotte Auerbach, Institute of Animal netics, Edinburgh, Scotland.

[6] R. F. Kimball, Oak Ridge National Laborat Oak Ridge, Tennessee.

od is usually plotted, and this may be
leading if the curves are interpreted
nean that the changes in the mutation
cess are really confined to this period.
his connection, I would like to ask Dr.
kin what are the possibilities that some
her decline curves reach a lower limit
ause the combined effects of the two
ods reduce premutational damage to
wer, irreversible limit, not because this
it is reached during the first interval.

WITKIN: It is certainly true that the
ire period between exposure to radiation
duplication of DNA must be consid-
l in any postirradiation influence on
tation frequency. In the case of the
line curves presented in this paper, I
not think that the period between plat-
out and DNA duplication contributes
he reduction of the mutation frequency,
ce the plating medium used was one
own to support a high yield of induced
tations. Further evidence that the de-
e in mutation frequency is accom-
hed during the interval of treatment
he fact that the same low yield is ob-
ned whether the final plating medium
nriched with amino acids or not.

IEINMETS[7]: I have a question about
s. Was this experiment being done
the dark or under anaerobic conditions
l has there been any attempt made to
nove dyes by ionic substitutions? (This
estion is related to phytodynamic ac-
n.)

WITKIN: The experiment was done in
dark, not anaerobically. The only re-
val of the dye was with nucleate rever-
. Otherwise, the posttreatment was
led by diluting out of the dye tube and
re were no residual effects of the dyes.

HEINMETS: Has dye effect been tried in
ar medium?

WITKIN: The posttreatments in which
dyes were tested for their effect on the
s of mutations were given in liquid. The
cteria were maintained in dye-contain-
liquid and plated out at various times.

LIEB: Is there any evidence there is a
by error, which would give rise to mixed
nes of mutants? If we study the in-
ease in the parental cells and in the mu-
ts after UV, the parental cells—the number of cells per volume—will show a short lag and then go up logarithmically.

The frequency of mutants assayed on semi-enriched plates will show a longer lag and then go up at the same rate so the mutants do not have a different rate of growth.

If we study the difference in lag we find that when the mutant frequency starts to increase, the non-mutants have increased by a factor of 4; this I think supports segregation of mutants.

WITKIN: I think it is not possible in that kind of experiment to distinguish between segregation of multiple nuclei and segregation of components of single genomes. It is necessary to control nuclear multiplicity before such data can be useful.

LIEB: The number of stationary phase B/r is two so far as we can see them in stained preparations.

WITKIN: There are ways of studying this with an absolute control over the nuclear multiplicity which I think will give a clearer answer.

R. **SETLOW**[8] : Apropos of the number of nuclear bodies, I should say there exists radioaudiographic evidence that there are four large DNA structures in *E. coli.*

LIEB: At what stage of the culture?

R. **SETLOW**: Exponential growth.

LIEB: And at stationary phase?

R. **SETLOW**: Two.

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ucleic Acid Formation and Ultraviolet Light-Induced utation in Bacteria: Some Considerations Light of Recent Advances

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Houston, Texas

n the ultraviolet light (UV) induced
ersion of certain auxotrophic strains
Escherichia coli, the terminal process
ough which the genetic change is in-
porated into the genetic apparatus ap-
rs to be the initial postirradiation syn-
sis of deoxyribonucleic acid (DNA).
ee lines of evidence support this view.
he first place, loss of photoreversibility
orrelated with the progression of DNA
thesis (Haas and Doudney, '60; Doud-
and Haas, '60), suggesting that DNA
thesis is the copying event leading to a
tostable "error" in the genome. Sec-
ly, mutation expression occurs only
r DNA synthesis (Haas and Doudney,
). Furthermore, expression requires
tein synthesis after DNA synthesis
as and Doudney, '59). Finally, the ab-
ce of thymine with consequent block-
of DNA synthesis prevents expression
amino acid independence in thymine-
and amino-acidless polyauxotrophs
oudney and Haas, '60, unpublished
a). It thus appears clear that the func-
al mutated gene is formed with DNA
lication.

At the time that Dr. Witkin ('56) re-
ted her initial studies in this area, Dr.
ix Haas and I were studying UV in-
ed mutation using a different approach.
found that supplementation with the
ines and pyrimidines occurring in ribo-
cleic acid (RNA) markedly increased
mutation frequency of "color variants"
eosin-methylene blue agar (Haas and
udney, '57), suggesting the hypothesis
t nucleic acid precursors altered *in vivo*
UV might serve as mutation inducing
nts when subsequently incorporated
o nucleic acid.

On learning of Witkin's results, we turned to studies of postirradiation processes (Doudney and Haas, '58; '59; '60). We suggested the term "mutation stabilization" for those interactions dependent on amino acids and increasing or supporting mutation prior to any net measurable macromolecular synthesis (fig. 1). It is our view that the effectiveness of mutation stabilization determines mutation frequency under ordinary conditions. Thus, in this special sense, the original view of Witkin ('56) that protein synthesis determines the level of mutation induction appears to be valid. Although the nature of mutation stabilization is not known, it is quite attractive to interpret it as a templating or preparatory process to gross macromolecular synthesis in the UV-exposed cell. The evidence further suggested that, if mutation stabilization does not occur, an antagonistic or competitive process occurs which removes the potential mutation from pathways leading to mutation induction (Doudney and Haas, '59). This process we have termed "mutation frequency decline." If one assumes that the potential mutation resides initially in the DNA existing at the time of UV irradiation, then the process of decline may be ascribed to repair of the UV damage in DNA. If the alternate view is taken that the potential mutation resides in a UV modified nucleic acid precursor, then any process removing this precursor from the synthetic pathways of nucleic acids involved in genetic replication would prevent mutation. Whatever the nature of the process of mutation frequency decline, it is evident that this enzymatic process can be carried to completion in the absence

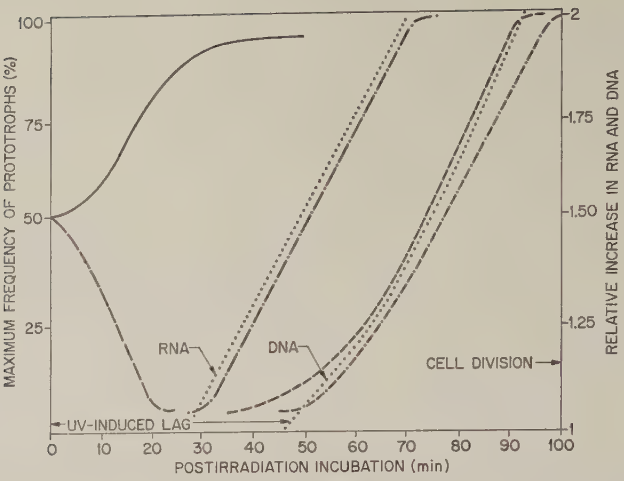

Fig. 1 The time course of several operationally-defined postirradiation processes of mutation induction by UV in relation to the synthesis of RNA and DNA in the culture. —, Mutation stabilization is determined by incubation at 37°C. in an amino acid-rich medium after UV exposure and then plating at the indicated times on minimal agar medium containing 2.5% nutrient broth. – – –, Mutation frequency decline is determined by adding 6-azauracil, 50 μg per ml, or chloramphenicol, 20 μg per ml, to the culture in the above amino-acid rich medium immediately after UV exposure, followed by incubation of the culture for the indicated times prior to plating on the nutrient broth containing agar medium. · — · — ·, Mutation fixation is determined by adding chloramphenicol or 6-azauracil to the culture after incubation in amino acid-rich medium for the indicated periods of time and then incubating in the presence of the antimetabolite for an additional 30 minutes prior to plating on nutrient broth supplement agar medium. - - -, "Mutation incorporation" is visualized by incubation of samples for the indicated periods of time after exposure in the amino-acid containing medium and then exposing them in the cold to intense white light for one hour prior to plating (photoreversal challenge). · - - · - - ·, Mutation expression is measured by plating on unsupplemented minimal agar medium after incubation in amino acid-rich medium containing the required amino acid. Incubation on agar medium is for 3 days at 37°C. All these experiments were carried out with *Escherichia coli* strain WP2, a tryptophan-requiring strain, and reversion of this requirement is followed. Comparable results have been found with several other strains. UV dose resulted in about 50% survival and was measured as about 900 ergs per mm². Cell division begins at about 100 minutes and the number of colony-forming organisms doubles in 10–15 minutes. The basic techniques used are described by Doudney and Haas ('60).

of RNA, DNA and protein synthesis and without influencing materially the timing or rate of subsequent macromolecular syntheses definitely involved in mutation induction (Doudney and Haas, '60).

What happens to the potential mutation if mutation stabilization takes place rather than mutation frequency decline? We demonstrated that postirradiation incubation in medium containing amino acids led to fixation of the potential mutation in the sense that agents that block RNA or protein synthesis would no longer promote mutation frequency decline, after some synthetic event had occurred (Doudney and Haas, '58). We expected that this synthetic event would turn out to be DNA synthesis. However, we established th
mutation fixation is correlated with t
initial doubling of RNA in the cultu
(Doudney and Haas, '59). Studies wi
thymineless amino-acidless strains indica
that mutation fixation proceeds in t
absence of thymine. This is in harmo
with the findings of Harold and Zipor
('58) and Doudney ('59), that recove
from the UV-induced block to DNA sy
thesis occurs in the absence of thymine
a thymineless strain. A relation has be
established between the amount of po
irradiation RNA synthesized at the time
chloramphenicol addition and the rate
DNA synthesis in the presence of chl
amphenicol (Doudney and Haas, '60

ese studies suggest that only where some
A and protein moiety necessary to and
e limiting for genetic replication is
med, prior to chloramphenicol addition,
ı DNA be produced with consequent
ablishment of mutation in the genome.
this RNA and protein has not been
med at the time of chloramphenicol
dition, the potential mutation is lost
ough the process of mutation frequency
cline. It seems likely that the RNA and
tein involved in mutation fixation is
ntical with that involved in the post-
adiation recovery of the DNA synthe-
ing mechanism.

Recently, we have been studying the
ect on mutation induction and DNA syn-
esis of certain analogues which may be
corporated into RNA to interfere with
acromolecular function. Among these
alogues, 5-hydroxyuridine has proved to
the most interesting and useful (Doud-
y and Haas, '59; '60). 5-Hydroxyuridine
s the interesting capacity to block the
rmation of the adaptive enzyme, β-galac-
idase, while allowing RNA and protein
nthesis to continue (Spiegelman, Halvor-
n and Ben-Ishai, '55). We have shown
at this analogue produces a decrease in
mutation frequency in correlation with the initial doubling of RNA in the culture (fig. 2). The most reasonable hypothesis to explain both of these effects is that the incorporation of 5-hydroxyuridine into RNA results in biologically modified RNA which leads, in one case, to non-enzymatic protein and, in the other, to non-functional or non-genetic DNA. At least, with this uracil analogue system, we can study relevant changes in DNA resulting from analogue incorporation into RNA on both a macromolecular and genetic basis, with some hope of getting significant answers.

Considerations of this sort, of course, led us to the expression of our rather unorthodox hypothesis for DNA replication and mutation induction (Doudney and Haas, '58; '59). According to this hypothesis, DNA synthesis (at least after UV exposure) involves transfer of information from the parental DNA to the daughter DNA through an RNA-protein intermediate. On this basis, incorporation of a UV-modified RNA precursor into the single-stranded RNA would lead to a copying error involving the substitution of a different nucleotide pair in the subsequently formed daughter double-stranded DNA.

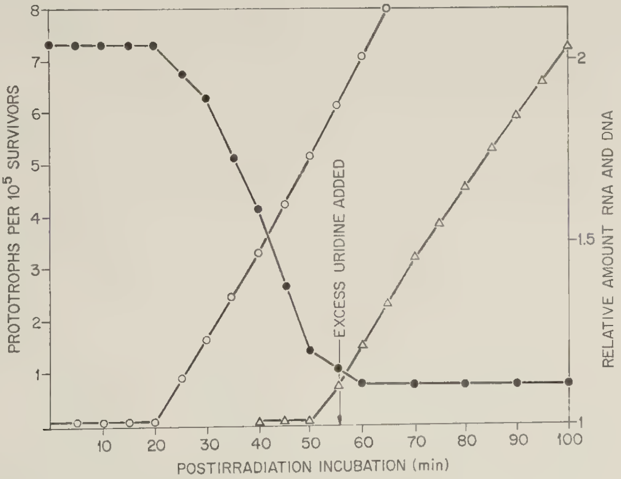

Fig. 2 The effect of 5-hydroxyuridine on the yield of prototrophs from *Escherichia coli* strain WP2 with postirradiation incubation. The 5-hydroxyuridine was added immediately following UV exposure to the culture in a minimal medium supplemented with a rich supply of amino acids including tryptophan (Doudney and Haas, '60). Plating on minimal agar medium supplemented with 2.5% nutrient broth was at the indicated times. Uridine (50 μg per ml) is added after 55 minutes incubation so that any 5-hydroxyuridine effect on DNA synthesis is reversed. The techniques used are described by Doudney and Haas ('60). ●, mutation (+ 5-hydroxyuridine); ○, RNA (+ 5-hydroxyuridinc); △, DNA.

If the Watson-Crick hypothesis for DNA replication holds after UV exposure, then, of course, we must adapt our theories to this model. It is not mandatory that we abandon this hypothesis to rationalize our data. Rather, it may be that this direct mechanism for DNA replication actually holds after UV exposure, but that the RNA-protein component which our data indicates plays an intimate accessory role to DNA synthesis, so that a UV-created mutagen incorporated into the RNA could modify the newly formed DNA.

What we need to understand now is the molecular mechanism of DNA replication following UV exposure. Our studies and the studies of others leave little doubt that the synthesis of some RNA and protein moiety is requisite to DNA synthesis in UV exposed cells (Harold and Ziporin, ’58; Doudney, ’59; Drakulic and Errera, ’59; Doudney and Haas, ’60). Further, recent work by Doudney and Billen (’61) seems to eliminate the possibility of the enzymes, DNA polymerase or thymidine kinase, being involved in the UV-induced block to DNA synthesis. Exposure of cultures to energy levels of UV far above those required to block completely DNA synthesis *in vivo* does not decrease the thymidine-incorporating activity of cellular extracts. Furthermore, a marked increase in these enzymes occurs during the period of blocked DNA synthesis. Thus we must look elsewhere than the Kornberg enzyme for the RNA-protein moiety involved in DNA replication and mutation induction following UV exposure.

If there is no effect of doses of UV that block DNA synthesis on polymerase, then very possibly the *in vivo* UV induced block is caused by a UV effect on the “primer,” that is to say, the cellular DNA. It is known that *in vitro* DNA synthesis requires that the “primer” be in the single-stranded condition. If this is the case in the cell then the *in vitro* effect of UV on DNA described earlier by Dr. Marmur might underlie the UV-induced block to DNA synthesis. According to Dr. Marmur, the strands of DNA *are held together* by some photochemical effect of UV absorption. We might then assume that DNA synthesis is prevented by UV because the strands of DNA cannot be separated by cellular mechanis to make them available as “primer” f action of the Kornberg system *in vivo.*

Logically, then, the recovery of DN synthesis after UV exposure (throu RNA and protein synthesis) would ha to be due to one of two mechanisms. T RNA and protein formed could be involv in repair of the UV-induced lesions whi hold the single strands of DNA togeth On the other hand, the cell may posse an alternate mechanism for DNA replic tion, when the direct replication mech nism is blocked, involving transfer of i formation from double stranded DNA some intermediate RNA-protein structu and back to daughter DNA. This seco hypothesis seems the more plausible of t two in light of our mutation studies, esp cially those with 5-hydroxyuridine. W have suggested that most of the mutati induction data, as well as the necessity f RNA and protein synthesis in DNA replic tion following UV exposure, could be a counted for by the assumption of such alternative mechanism of DNA replic tion following UV exposure (Doudney a Haas, ’60).

According to this model, chloramphen col-sensitive UV induced mutation wou depend on two radiation effects: (1) bloc age of DNA synthesis by UV, causing transfer of information through an RN protein intermediate, and (2) the esta lishment of the photochemical modific tion which eventually results in mutati (presumably the UV-modified nucleic ac precursor). These UV effects clearly a not identical. Chloramphenicol or oth agents that interfere with RNA or prote synthesis prevent mutation but do not r verse the UV-induced block to DNA sy thesis. According to this model, the indu tion of the alternate mechanism of DN replication by UV is a necessary pr requisite to the chloramphenicol-sensiti mutation mechanism. Mutation then wou result through the incorporation of t modified nucleic acid precursor into t intermediate RNA strand.

We suggested that photoreversal of m tation is due to an effect of white light the photochemical change (the UV mo fied precursor) which leads to mutati udney and Haas, '60). However, it ns more likely (especially, in view of Rupert's results on photoreversal of induced lesions in DNA via an enzyic mechanism) that the prevention of ation induction by white light is due the photoreversal of the UV-induced k to DNA synthesis. This reversal of block would allow the return to direct A replication via the Kornberg system result in bypassing the mutagenic hanism based on the formation of intermediate RNA-protein structure. It ld be expected, according to this model, t photoreversal would be successful in venting mutation by causing a switch k to the direct replication mechanism, until the time of DNA replication via alternate mechanism. This possibility n agreement with experimental findings aas and Doudney, '60; Doudney and as, '60).

similar effect on "choice" between alnate DNA replication mechanisms may involved in the mode of action of cafe and the DNA-combining dyes in inasing the frequency of mutations, as cribed earlier in this conference by Wit. The mode of action of these agents y be to help bind the DNA strands toher at dose levels of UV near the thresh of the binding effect and thus to inase the frequency of adoption of the tagenic alternate mechanism of DNA lication in the cell population.

How might the alternate mechanism of A replication have come about? Of rse, it could be the primitive mecham. We have pointed out the attractives of this indirect mechanism of DNA lication from the viewpoint of undernding the evolution of heterogenetic ivity from the replicative process (Doudy and Haas, '59). According to this w, the Kornberg system evolved later a more efficient and advantageous chanism which is less subject (obvisly) to the deleterious effects of too freent mutation induced by UV from the n. The primitive mechanism might have en retained, however, because cells possing this mechanism in reserve in addin to the Kornberg mechanism might joy a selective advantage, in life in sunlight. These cells might meet more successfully the ages-long challenge to the Kornberg system by sunlight (based on UV-induced bonding of the strands), by being able to resort, as the environment demanded, to the primitive mechanism. On the other hand, perhaps the indirect mechanism is an evolved reserve mechanism of DNA replication in cells whose Kornberg system of DNA replication was constantly being challenged by the sun.

ACKNOWLEDGMENTS

The author wishes to acknowledge the collaboration of Dr. Felix Haas in the mutation induction studies and Dr. Daniel Billen in studies of enzymatic incorporation of thymidine in bacterial extracts. This work was supported by the Atomic Energy Commission and the National Institutes of Health.

OPEN DISCUSSION

KADA[1] : I think that the main differences between the speakers concerns the mutational sites of irradiation and the model of DNA replication following UV irradiation.

We have some results that might throw some light on these considerations. The experiments are concerned with mutation response to a second irradiation of bacteria that have been primarily irradiated and incubated for varying periods of time.

The strain used was *E. coli* B/r WP2 try$^-$ grown overnight in a synthetic medium. This culture was irradiated with UV and incubated in the presence of a complex supply of amino acids plus tryptophan. The cells were washed at different times of incubation, irradiated again with UV and plated on minimal agar plates without tryptophan. The portion of mutations induced by the second irradiation, after subtraction of those induced by first irradiation, increased remarkably with incubation and in the absence of DNA synthesis in the earlier incubation periods, and then decreased abruptly at about the time when the amount of DNA doubles before cell division.

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This disappearance of such a mutation response prior to the time of DNA replication is difficult to interpret by the supposition that the observed mutation response does not relate to increased genetic change, but concerns the modification of degree of expression of the mutations since expression is only observed after DNA formation. Rather it would appear that a certain constant proportion to total mutations is always expressed on minimal medium and that we are following an increase in mutation frequency response by observing the increase of the quantity which is expressed on minimal.

If this is really so, the increase in mutation induction by the second irradiation observed here is not correlated with DNA synthesis, but rather with some capacity which is formed prior to DNA synthesis. The hypothesis proposed by Dr. Doudney is convenient to explain these observations. Thus, some special RNA-protein structure intermediate in DNA synthesis is formed after UV exposure which, when irradiated, results in increased mutation upon formation of DNA. We have found, incidentally, that chloramphenicol blocks the formation of the increased capacity for mutation response.

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me Postirradiation Phenomena that Affect the duction of Chromosome Aberrations

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or at least 34 years now it has been ain that radiation can bring about etic changes. Those that were true e mutations, it was believed, were sed by a simple physical interaction of radiation with the gene, and that, con- uently, these events were final at the ment of such interaction. There exists, ever, another class of radiation-in- ed mutations, the so-called intergenic ations, or aberrations, that come about n the breakage and subsequent rear- gement of the chromosomes. In this er, we will examine the thesis that the etic effects of radiation detected as omosome aberrations are the conse- nce of localized chromosomal lesions, are subject to modification after ir- iation.

he concept of localized and reparable ons has been long established for omosome aberrations, having been ed clearly by Stadler as early as 1932, goes back to the early development he breakage-first hypothesis during the 0's (see Muller, '54, for a history of). It was firmly established by the neer work of Sax ('40, '41) on *Trade- ntia* and was formulated in consider- e detail in Lea's book ('46). The main clusions were that ionizing radiations ke the chromosomes and that the broken ds then could either reunite with ends m other breaks to form exchanges, or titute by rejoining in the original con- uration. It was soon realized that the at majority of breaks undergo restitu- al rejoining and, as suggested by most the evidence, that the repair by restitu- is complete (Lea, '46). Whether or there were also some breaks that were ejoinable and so nonreparable has been subject of considerable speculation.

Chromosome aberrations

Until 1954, rejoining was generally believed to be a physical process, and was described by the simple terms we have used—rejoining, reunion, and restitution. It then became apparent to us, however, that when breaks rejoin, stable molecules are formed in the sense that the rearranged chromosomes are able to persist in their new form. Since this was the case, we postulated that the rejoining was not a simple physical fusion of the ends but came about by the formation of chemical bonds (Wolff and Luippold, '55). If this assumption were correct, then we expected the process of rejoining to require energy and even perhaps an enzyme, as was necessary for almost all biosynthetic processes that occurred in the cell. In spite of the fact that we do not know exactly how a chromosome is constructed, it was possible to attack these problems with the use of metabolic inhibitors.

Since the aberrations observed are the result of at least two processes, breakage and subsequent rejoining of breaks, it was impossible to demonstrate rigorously that the effect of an auxiliary treatment with radiation was on only one of them to the exclusion of the other. In other words, a treatment that would decrease the numbers of visible aberrations could conceivably either decrease the numbers of breaks or increase the amount of restitution. In order to escape from this dilemma we resorted to dose-fractionation studies on two-break aberrations, which increase as the square of the dose (Wolff and Luippold, '55).

The rationale for such experiments is that if the radiation is broken into two doses separated by a time sufficient for all of the ends to rejoin, then the yield of aberrations will be the sum of those aberrations produced by the breaks from the first dose rejoining with themselves to form dicentrics and rings plus the aberrations produced from the breaks of the second dose rejoining among themselves. If, however, after the breaks have been induced with the first dose, something is done to inhibit their rejoining so that they will be open and capable of rejoining after the breaks from the second dose have been produced, then the breaks from the two doses will be present concurrently, able to rejoin with each other, and the yield will be proportional to the square of the total dose administered (Wolff and Atwood, ’54).

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[1] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

Metabolism and rejoining

By such experiments, using metabolic inhibitors, we established that the rejoining of radiation-induced chromosome breaks was indeed subject to metabolic control. For instance, low temperatures that would stop enzyme activity, cyanide that would inhibit many enzymes within the cell, carbon monoxide that would inhibit cytochrome oxidase activity in the dark but not in the light, and dinitrophenol that would uncouple oxidative phosphorylation were all found to prevent rejoining. It was found, conversely, that the addition of exogenous adenosine triphosphate (ATP) stimulated the rejoining of the chromosome breaks (Wolff and Luippold, ’56a, b).

In an attempt to further define the bonds that were being formed when breaks rejoin, we carried out similar experiments with protein synthesis inhibitors (Wolff, ’59, ’60). These were also found to prevent rejoining of the breaks. As a result of the earlier experiments, in which it was found that metabolic inhibitors prevented the rejoining of breaks that ordinarily remained open for long periods of time and that ATP stimulated the rejoining of these breaks, it was postulated that the bonds formed during rejoining were covalent (Wolff and Luippold, ’56b). As a result of the work with protein inhibitors, we went further and postulated that the X ray-induced breaks were in the protein moiety of the chromosome and that protein or perhaps
more simply peptide links were synthesiz
when the breaks rejoined (Wolff, ’60).

It should be pointed out that there a
other points of view in regard to the natu
of the breaks induced by radiation. St
fensen (’55), for instance, favors the h
pothesis that the chromosome is held
gether by weak secondary bonds and th
X radiation primarily attacks these plac
In addition, Revell (’59) has resurrect
the old contact-first hypothesis. He post
lated that the radiation first induces ac
vated states in the chromosomes that if
contact with one another can at some lat
time give rise to an aberration by a brea
age and rejoining process akin to crossi
over. This hypothesis, however, oper
tionally leads to results that are near
indistinguishable from those expected
the breakage and rejoining concept ge
erally held by cytologists and geneticists.

In any case, what has been definite
established is that the radiation-induc
damage is not absolute and final at t
moment of irradiation, but is subject to
dissipation or repair in the postirradiati
period and that this repair is dependent
the metabolism of the cell. As pointed o
above, we believe that our experiments gi
some indication about the nature of t
bonds formed when breaks rejoin an
consequently, give some clues to the stru
ture of the chromosome.

Amount of restitution

In the study of chromosome aberration
it soon becomes apparent that, because
restitutional repair, we are able to obser
only a fraction of the breaks originally i
duced in the cell. What we really mu
study in order to gain information abo
the repair processes is not just those brea
that remain to give visible aberrations b
all of the primarily induced breaks withi
the cell. Lea (’46) estimated that f
Tradescantia some 90 to 95% of all p
mary breaks restitute.

In order to study these, we have resorte
to a method devised by K. C. Atwood f
determining the numbers of primary brea
necessary to give a certain yield of tw
break chromosome aberrations. The calc
lations, which are presented below, ha
led us to conclude that more than 99%
all breaks restitute and that there are on

nited number of places—referred to as s" (Wolff, '59)—within the interphase leus where the chromosomes happen to e close enough to one another for re- ing other than restitution to occur. se same calculations indicated that the erences in sensitivity to radiation ob- ed at different stages of cell division not manifest at the molecular level and the chromosome strand has essentially orm sensitivity throughout the cycle. ge sensitivity, therefore, resolves itself question of the availability of other aks within the distance, h microns, which a break can rejoin with another ken end. In some stages of division, e will be more of these places within nucleus; consequently, there can be e rejoining that will give rise to visible omosome aberrations than at other ges. If the breaks cannot rejoin in this ion they will restitute and so not be arent. This, of course, implies that the air process for chromosome aberrations s on at all stages of the cell cycle as been known for a considerable time. s fact alone is enough to make us think erms of protein synthesis rather than xyribonucleic acid (DNA) synthesis in rejoining of the breaks, since DNA thesis occurs only during a limited part he cell cycle.

he calculations utilized expressions for yield of two-break exchange aberra- s induced by a dose of neutrons or X s (Wolff *et al.*, '58; Wolff, '59) in which yield was expressed in terms of the ance h over which an exchange can ur and of the numbers of primary aks induced in the cell. The rationale the expressions is that if a given dose neutrons with a total path length of d rons produces n_1 breaks, then the prob- lity that a given break will be in any en micron of path length will be 1/d. probability that another break will be hin the distance h over which an ex- nge can take place will be 2h/d since second break can occur within h mi- ns on either side of the original one. s is the probability that each of the $(n_1 - 1)]/2$ pairwise combinations of n_1 breaks has of being a potential exchange. The yield will then be approximated by the formula

$$Y_1 = \frac{hn_1(n_1 - 1)}{d}$$

For X rays, the formula is somewhat different with the yield

$$Y_2 = \frac{h^3 n_2 (n_2 - 1)}{2R^3}$$

where R is the radius of the nucleus. In the X-ray case, the first break can be anywhere within the volume of the nucleus and the second break can then be induced anywhere within a sphere of radius h from the original break. Therefore, the probability of two breaks being within this sphere will be proportional to the ratio of the volume of this sphere to the volume of the nucleus. In table 1 we show the values for the n breaks induced with X rays and neutrons at doses that gave a yield of 0.1 exchanges per cell calculated when h varies from 1.0 to 0.1 μ. It may be seen that with a smaller h, the number of breaks induced for a given exchange value not only is larger in both cases but is relatively larger with X rays. This is what would be expected from the kinetics of exchange production in that the target of radius h has to be hit only once with the protons projected by neutrons in order to get an exchange but has to be hit twice with X rays. As the target becomes smaller and harder to hit, more breaks will have to be produced in the X-irradiated cells in order for the target to be hit twice. It should be pointed out that the estimate of n from the equation is a minimal estimate since not all of the potential exchanges are realized.

TABLE 1

The numbers of breaks necessary to produce a yield of 0.1 chromosome exchanges per cell

h	Neutron n_1	X ray n_2	n_2/n_1
1	5.78	7.64	1.32
0.5	7.68	19.6	2.55
0.4	8.45	27.0	3.20
0.3	9.59	41.0	4.27
0.2	11.5	75.5	6.57
0.1	15.8	206.0	13.0

From the results of combined X-ray and neutron experiments, the distance h was calculated to be about 0.3 μ (Wolff *et al.*, '58).

Other calculations for the numbers of sites within the nucleus where the chromosomes come within the exchange distance, h, were made by Atwood and myself (unpublished). These indicated that there were only, on the average, 4 sites within a *Tradescantia* microspore nucleus. Having this value, we were then able to make a more accurate estimate of the value of h.

The method for the estimate of the number of sites is based on an observation by Atwood and me (Wolff, '59) a few years ago that one group of aberrations, the chromosome exchanges, didn't fit a Poisson distribution of $e^{-m} \cdot m^r/r!$ where r would be the number of exchanges in the cell and m would be equal to the mean number per cell observed in an experiment. There were always too few cells having multiple aberrations which made us think in terms of a small number of sites.

Von Borstel suggested a method whereby it was possible to solve for the number of sites. Although the method could give only an approximation of the site number, it did give the proper value as could be checked by other calculations.

The method is analogous to a two-hit survival curve analysis. If the cell has several sites where two chromosomes, if broken, are close enough to form an exchange, the chance of not getting a break in one of the sites in a cell would be the first term of the Poisson distribution or e^{-m}.

The chance, therefore, of hitting and breaking a chromosome at least once in the site where the chromosomes come together would be $(1 - e^{-m})$. But in the X-ray case, two hits are necessary to break both chromosomes and form an exchange. The chance of getting both hits is the product of the chance of getting each of the individual hits. Assuming the sensitivity is the same for both hits, the product will be $(1 - e^{-m})^2$.

This then is the chance of getting an exchange at a site. The chance of not getting an exchange at a site is $1 - (1 - e^{-m})^2$. The chance of not getting an exchange at all sites and therefore in the whole cell is the product of not getting it at each of the n possible sites. If the sensitivities at all sites are the same this product will be $[1 - (1 - e^{-m})^2]^n$.

Using Alan Conger's extensive data fr
operation Greenhouse material, we
tained the best fit to this last expressi
For *Tradescantia* n came out to be 4, a
the fit was an excellent one.

A Poission distribution of aberratio
would be expected only with an infin
number or a very large number of pla
where exchanges could take place; w
only 4 sites the distribution might be
pected to be not Poisson but binomial.
then in an experiment the observed me
number of exchanges per cell was m, th
the mean number per site would be m
Cells with no exchange would have to
free of exchanges at all 4 sites. Cells w
only one exchange could have an exchan
at only one of the sites and so forth.

The probabilities for these numbers
exchanges are given by the binom
expansion $[(1 - m/4) + m/4]^4$. If the
frequencies are used to compute the
pected values, the observed distributi
does not differ from the expected.

The number of sites can also be fou
without obtaining a whole dose curve
taking the data, breaking it down into t
cells without 0, 1, 2, 3, etc., exchanges a
seeing what power binomial it will fit
that is, testing it against the binomial
pansion $[(1 - m/n) + m/n]^n$ where n
the number of sites. With this meth
wherein the data is divided into all
classes, more information is gained th
from the survival curve type of analy
wherein only the zero class is used.

In our experiments with *Vicia,* the s
number was two and in *Tradescantia*
was 4. Evans ('62) has presented data
Vicia that is very close in this respect.

Knowledge of the number of sites th
allowed us to proceed with what we co
sider to be a more accurate estimation
the value of h. The method utilized is
follows: In the neutron case, there a
n_1 breaks induced at random within t
cell; twice the yield or $2Y_1$ is the number
these breaks at sites because each aber
tion comes from two breaks. Since brea
are distributed randomly throughout t
genome, this then means that $2Y_1/n_1$
equal to the proportion of the genome th
is at sites. Therefore in the X-ray ca
where n_2 breaks are induced in the ce
$2Y_1n_2/n_1$ is equal to the number of X-r

ks that are at these sites. We may call number X. The X-ray yield, Y_2, will be l to the number of X-ray sites that two breaks in them and therefore $2Y_2$ will be essentially equal to the ber of X-ray sites that have only one k. Y_2 then will be proportional to P^2 e P is the probability of breaking a mosome in a site. $2P(1 - P)$ will be ortional to the number of sites with one break in them or $X - 2Y_2$. Since e are only 4 sites, the frequencies obed from the binomial expansion are iplied by 4 to get the number of sites 0, 1, or 2 breaks. That is, $4P^2$ will l Y_2 and $4[2P(1 - P)]$ will equal the ber of sites with only one break. The r is $X - 2Y_2$. Since we know Y_2, we have the value of X which is in terms of Y_1 which we know from experimental results and the ratio n_2/n_1. It may be seen in table 1 that the proper ratio for n_2/n_1 which is 6.35 is not approached until h is equal to 0.2 μ.

Straightforward target-theory calculations for the numbers of cells not having an exchange after a dose of neutrons have also led to a similar value of h, in this case 0.18 μ. Consequently we have settled on 0.2 μ as being the true value.

As may be seen in table 1, in the case of X radiation at this value of h, there are some 75 primary breaks in the cell when the yield is 0.1 exchanges. In addition to the exchanges, other aberrations are also present. These include approximately an equal number of interstitial deletions and, we may assume, an equal number of un-

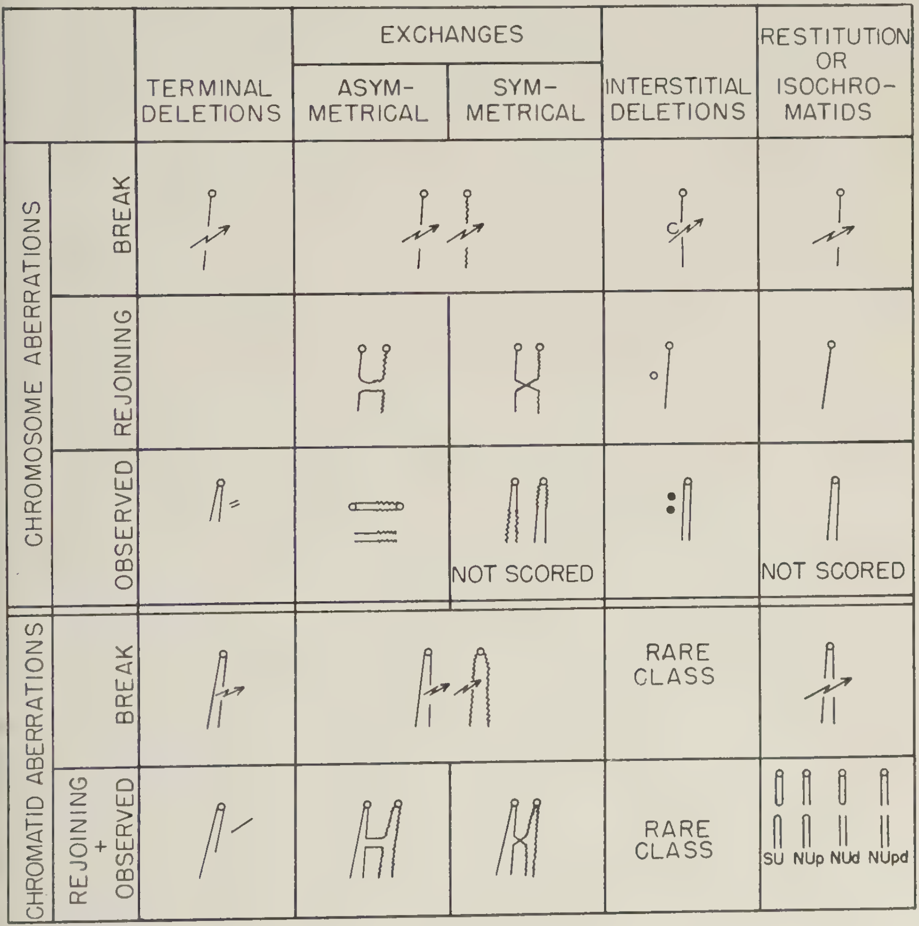

Fig. 1 Types of chromosome and chromatid aberrations commonly observed.

scorable symmetrical exchanges; also present are a very small number of perhaps true terminal deletions. All in all, this would indicate there are about 0.5 breaks per cell that result in visible aberrations from the original 75. The rest of the breaks have been repaired by restitution.

Stage sensitivity and repair

Since the repair process is so efficient, I began to wonder if the apparent increase in the sensitivity of the cells to aberration induction that occurs after chromosomal duplication might be caused by a decrease in repair. We were particularly interested in this because the experiments on mutations induced in *Paramecium* that are reported below indicated that, if anything, the repair was more efficient in this period.

A study was carried out in which we irradiated *Vicia faba* root tips and also *Tradescantia* microspores and then fixed the cells at such times when we could observe either chromatid or chromosome aberrations. The chromatid aberrations are induced after chromosomal duplication, a relatively sensitive period; the chromosome aberrations are induced before duplication, a relatively resistant period.

As may be seen in figure 1, the types of aberrations scored are quite similar in the two groups. We score the asymmetrical chromosome exchanges and a similar group of asymmetrical chromatid exchanges. We do not score symmetrical chromosome exchanges because in most cases we cannot be certain that these have been formed, whereas we do score this class for chromatid aberrations. For this group then, we can score more in those cells in which we see chromatid aberrations, although an equivalent amount of damage might be present in the other cells. We score a group of interstitial deletions in the chromosome aberration group which we do not score in the chromatid aberrations and conversely a group of interchanges as chromatid but not as chromosome aberrations. We score terminal deletions and an equivalent group of chromatid deletions. There is, however, one great difference in the types of aberrations that can be scored in the two types of cells—that is, we can score for isochromatid aberrations in the chromatid group but not in the chromosome class.

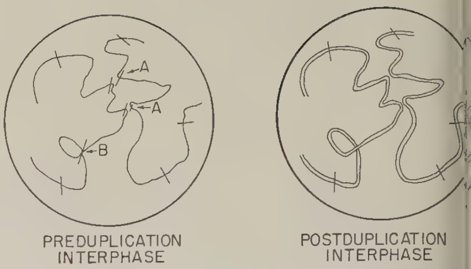

Fig. 2 Schematic diagram of pre- and p
duplication nuclei. Breaks at A are withi
microns of one another and able to form
changes. Breaks at B can result in intersti
deletion. Other breaks not being near ot
broken ends restitute. After duplication, th
other breaks have a sister strand within h
crons.

Figure 2 shows a schematic represen
tion of the interphase chromosomes with
the nucleus. It can be seen that there
only a few places (4 in *Tradescant*
where the chromosomes come within
microns of one another so that if brok
there an exchange can occur. These si
are marked A in the diagram. There
other places where the chromosome mig
loop so that breaks in these regio
(marked B) could give rise to interstit
deletions. Since the large majority
breaks are not in either of these types
places and since almost all breaks are
joinable (terminal deletions being a ve
small group if present at all) the v
majority of breaks will restitute.

After duplication, however, when ch
matid aberrations appear, the breaks th
would not have been at places where th
were within h microns of another bre
and so restituted now have a sister stra
very closely appressed to them. If t
sister strand is also broken, the end w
now have another type of rejoining ava
able rather than just restitution, and i
chromatid aberrations can be formed. T
majority of isochromatids show union
both pieces, the so-called sister union (S
type (fig. 1). A certain proportion sh
nonunion in the proximal segment (NU
but have union in the distal portion a
another equal proportion show nonuni
distal (NU*d*). A far smaller group will
show any union at all, the NU*pd* grou
On such a scheme as this, we would
pect the difference in sensitivity in t
two groups of aberrations to come abo

TABLE 2

The percentage of chromosome and chromatid aberrations observed in Tradescantia microspores after 200 r of X rays. Chromatid aberrations observed 23 hours after irradiation; chromosome aberrations observed 4 days after irradiation

	Exp. 1		Exp. 2	
	Chromatid (350 cells)	Chromosome (400 cells)	Chromatid (300 cells)	Chromosome (300 cells)
Deletions (terminal?)	25.7	3.0	4.0	5.0
Exchanges	26.8	17.8	24.7	15.7
Interstitial deletions	—	14.5	—	10.0
Isochromatid deletions	43.1	—	50.0	—
Intrachanges	16.0	—	18.0	—
Total	111.6	35.3	96.7	30.7

nly because of what could be scored in
different types of cells and that by far
largest increase would come from the
ıp of breaks that ordinarily would res-
e but now can undergo another type of
ining resulting in visible isochromatid
rations. Table 2 shows just such a
tion in which the numbers of ex-
nges are about twice as frequent in the
matid group as in the chromosome
ıp as would be expected, since we only
e the asymmetrical exchanges when
score for chromosome aberrations, but
e for both asymmetrical and sym-
rical ones in the chromatid aberrations.
he first experiment our class of chro-
id deletions is seemingly greater than
numbers of terminal deletions induced
he cells. Revell ('59), however, has
wn that the class of chromatid dele-
s is really a heterogeneous group, most
which are but simple achromatic gaps
are not true breaks at all and that the
nber of true chromatid deletions is very
ll and akin to the numbers of terminal
tions observed in cells showing chro-
some aberrations. In the second experi-
nt only true breaks were scored as
matid deletions and they were found
ccur only as frequently as did chromo-
e terminal deletions. This, then, leaves
further class, the isochromatid aber-
ons, that come from breaks that ordi-
ily would restitute. These now are able
ontribute almost 50% of the damage.

t, therefore, appears as though the re-
mechanism is operative throughout
cell cycle and that the differential sen-
vity of the stages of mitosis is really not
fferential sensitivity of the chromosome
simply a reflection of the fact that more of the primary breaks can rejoin to form visible aberrations after the chromosome has duplicated than before.

In addition to providing an explanation for stage sensitivity, the concept of a limited small number of sites within the nucleus where breaks are close to one another has allowed us to account for the distribution of chromosome exchanges within cells. It is also possible for us to explain on this basis such other phenomena as the apparent lack of rejoining between breaks induced by different chemicals that act on fairly specific loci on the chromosome as described by Merz *et al.* ('61). In order for breaks caused by two such different chemicals to rejoin, the specific regions attacked would have to be within h microns of one another. Since there are such a small number of places within the nucleus where the chromosomes in general are this close, the probability of it happening for specific regions is very low. X-ray breaks, however, which are more randomly distributed, can rejoin with the breaks induced by all of the different agents.

In summary, it can be said that our experiments on chromosome aberrations, whether they were biochemical or biophysical experiments, have indicated that the production of chromosomal aberrations is not necessarily fixed at the moment of irradiation, but that a repair mechanism operates in the postirradiation period. This repair mechanism is dependent upon cellular metabolism, energy production, and probably protein synthesis. Biophysical calculations in regard to the distribution of aberrations per cell and the numbers of primary breaks per cell have led to the conclusion that it is a very efficient process with more than 99% of the primary breaks being repaired. This repair process that is manifested as chromosome-break rejoining occurs throughout the cell cycle. Stage sensitivity of chromosomes can be accounted for in terms of this repair mechanism and the different spatial relations of the chromosomes and chromatids during the various stages of cell division.

OPEN DISCUSSION

LaChance[2] : Dr. Wolff, would you care to comment on the possibility that this distance h, the distance over which broken chromosome ends can reunite, is perhaps not a fixed inflexible distance, but is perhaps vulnerable to changes with temperature and other metabolic factors which might influence chromosome movement?

Wolff: Although we haven't particularly examined this point, in these experiments we have performed, such variations were not noticed.

Mukerjee[3] : Do you have any evidence for the formation of covalent bonds during the rejoining of breaks?

Wolff: The argument we make for covalent bonds rejoining is that the breaks ordinarily stay open for very long periods of time. If they were breaks of ionic links or something of that sort, we would expect them not to require energy, which we find is necessary, and not to stay open for long periods of time, but to snap shut. So we postulate that they must be covalent breaks.

Furthermore, when we work with chloramphenicol, or aureomycin, which are protein synthesis inhibitors, they too keep the breaks open which led us to think that perhaps this is the type of bond that is being formed when they do rejoin.

Abrahamson[4] : In *Drosophila* oocytes if one prevents restitution from occurring, we find we have increased the rearrangement frequency significantly.

We assume in this case this is due to the fact that the breaks are now kept open long enough to move around and interact with breaks with which they normally wouldn't interact.

Errera[5] : Just to remark on the interpretation of the effect of chloramphenicol—it is a well-known inhibitor of protein synthesis. I think one has to remember that in microorganisms, at le although it doesn't inhibit much of RNA synthesis, the RNA which is s thesized in the presence of chloramph icol is unstable. So we might be deal with some secondary effect due to the mation of unstable RNA in the absence protein synthesis.

Wolff: The only thing we did to try see whether or not chloramphenicol acting specifically on protein synthesis to put in C^{14}-labeled glycine, and then tract the proteins. We found that ind there was an inhibition of the incorpo tion of this label into the proteins.

There was no effect on P^{32} getting i the total nucleic acid pool, and DNA s thesis wasn't going on in the cells at So I think that this would indicate that least gross RNA synthesis or gross D synthesis wasn't involved at all.

Whether or not there is another type RNA synthesis going on or a new spec of RNA being formed we can't say becau we only looked at the total RNA that w produced.

Kaplan[6] : I think the same po could be checked more specifically w amino acid analogs, which I think perha should be tried in this system.

I also think I heard you say that si protein synthesis inhibitors interfered w restitution, presumably the breaks were protein. If you did say this, would you to defend it?

Wolff: The argument is that when get a break in a chromosome, metaboli and energy production is necessary for joining to occur. When we try to get m specific by using inhibitors, it turns that protein synthesis is necessary for joining to occur. This could possibly mea I think, a block of chromosomal protein made when a break rejoins. Or, m simply, a peptide link is made.

Now of course there can be some oth substance, be it what Dr. Errera is talki about or something else, that is made a is unknown. The formation of this su ce would be dependent on the *de novo* ation of an enzyme because the breaks narily stay open in the cell. They don't e unless protein synthesis occurs. The bition of protein synthesis could pre- t the formation of that enzyme.

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[2] Leo E. LaChance, U. S. Department of Agric ture, Kerrville.

[3] Debdas Mukerjee, University of Kentucky

[4] Seymour Abrahamson, University of Wisc sin.

[5] Maurice Errera, Université Libre de Bruxell

[6] Henry S. Kaplan, Stanford University.

also looks from the incorporation stud- as though this substance were not RNA DNA, which then tends to lead us to k more in terms of the proteins or the tide links.

APLAN: Let's go back to Dr. Rupert's e. If Dr. Rupert used chloramphenicol nis system he might well find that his toreactivating enzyme was not formed, which case a radiation-induced lesion, ch he can prove is actually in DNA, ld not be repaired.

One might draw the erroneous inference n this that the lesion was in protein, ch is not the case. I would say that it is least as attractive a notion to assume t your repairing system is an inducible , that it is in fact induced by the radia- n injury, and that the radiation injury y well be sustained in another kind of cromolecule, and my best bet would be A.

WOLFF: There, of course, might be ne inducible enzyme that is being med. What we are doing now is trying check if the bonds formed are protein putting labeled amino acids into the nu- i and the chromosomes. Of course the el then gets into the general proteins the cytoplasm and we have to extract chromosomes or the nuclei so that we n do audioradiography on them alone. is work is in progress now.

BIANCHI[7]: Rejoining phenomenon seems ated to moisture content and it can be served in seed when X-irradiated, rather n when treated with neutrons.

Can you comment, taking into account at happens in seeds, on rejoining phe- mena that you have described as oc- rring after treatments?

SWANSON[8]: May I have Dr. Conger re- ond to that?

CONGER[9]: At the present time I feel at the phenomena we are looking at in seeds and that Powers is looking at in spores and that Kirby-Smith is looking in the molecules bear no relation to the enomena of, say, after-effect and modifications that are seen in the normal wet metabolizing cells.

The evidence is simply this. All the biological, metabolic, biochemical factors that are so important in their phenomena—the time of DNA synthesis, the fact that further mutation or mutation reversal ceases when DNA synthesis occurs—doesn't apply at all to the dry state.

I think we are studying solid state reactions; they are studying biochemical and metabolic ones.

JACOBSON[10]: I have a little difficulty in understanding how you can break the chromosomes without breaking both the protein and the nucleic acid. Of course it might be a question as to which breaks first.

SWANSON: I think Wolff protected himself by saying he didn't know how a chromosome was put together.

JACOBSON: If you put the protein back together it would seem to me, regardless of what you are doing to the nucleic acid, you would be preventing rearrangement. This perhaps would be in defense of Dr. Wolff's position.

SWANSON: I think we must realize to go from something like DNA in its native naked pristine state to a chromosome is a tremendous jump. To make the switch back and forth is, I don't think, quite valid yet. I may be wrong, but I think there is a tremendous gap here in organization that we are not fully aware of. It is almost like comparing a bacteria to Sequoia so far as size is concerned.

JACOBSON: They both have DNA.

DANIELS[11]: Dr. Wolff, I wonder what effect, if any, the sol-gel state of the nucleus or the myxoplasm, which is a combination of the nucleoplasm and cytoplasm, might have upon rejoining? What effect do these various inhibitors such as chloramphenicol have upon changes in the sol-gel condition?

WOLFF: I am not quite certain just what the sol-gel relations are that we might expect to find in the interphase nucleus. The chromosome, of course, we know is the linear structure that has genes. Although invisible in interphase, the chromosomes enter it and come out essentially in the same position.

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[7] Angelo Bianchi, University of Milan.

[8] Carl P. Swanson, The Johns Hopkins University.

[9] Alan D. Conger, University of Florida.

[10] B. S. Jacobson, University of Texas, Austin.

[11] Edward W. Daniels, Argonne National Laboratory.

In addition, there is quite a bit of evidence indicating there is not much movement going on inside this interphase nucleus. So in our general approach, we haven't given much consideration to sol-gel relations at all.

I do know that we have used a great variety of reagents. Some of them, for instance, burn up the oxygen within the cell and others increase the oxygen within the cell. Some change the pH; some don't.

In spite of that, we find that the effects we get, the aberrations per unit dose with all these various pretreatments or post-treatments, be they low temperatures, cyanide, carbon monoxide, dinitrophenol, or simply complete anoxia, are the same. In addition the effect on rejoining is the same as we would expect in terms of their inhibition to metabolism.

Some of their physiological effects, pH and so on, you would expect to be different. Oxygen consumption is different. Cyanide, for instance, increases the intracellular oxygen concentration by inhibiting respiration so it is not burned out. Dinitrophenol, however, helps burn it up.

We can put in nitrogen and add a very strong reducing agent, sodium hydrosulfite, to take out oxygen and obtain the opposite effect from what is expected with cyanide. Yet these two work the same. It seems as though the important effect is the stopping of respiration.

So I am not sure any of these things do affect or might affect sol-gel relations and that if they did it would affect the breakage or the rejoining factors I am talking about.

SWANSON: There are, of course, great changes in the chromosome in terms of its water content as it goes through the various stages of its cycle.

ALEXANDER[12]: What is the shortest time interval that has been recorded between irradiation and the observation of permanent chromosome alterations (i.e., not stickiness).

SWANSON: Two minutes. This was done by Dr. David Bishop a long time ago in the spermatocytes of the grasshopper.

ALEXANDER: Were these physiologi alterations?

SWANSON: No, structural aberratio One thing that would interfere with seei them early is that they may regress stage, as Mary Esther Gaulden has show plus the fact that they get sticky—wh ever that means.

GRAY[13]: It is fairly easy to see how, the manner Dr. Wolff has shown us, wit holding energy between two fraction doses will increase the aberrations. Bu would expect Dr. Alvin Beatty to be sayi something if he is in this room, because has shown that withholding energy sourc immediately after a single short exposu when all the breaks are supposedly p duced simultaneously—if there are brea produced—increases the aberration f quency very considerably.

Now it is not so easy to see why wit holding energy should favor illegitima reunion against legitimate reunion—b no doubt Dr. Wolff can explain that rig away.

WOLFF: This is the same point to whic Abrahamson has spoken. In the *Drosop* *ila* system they see this quite regularl We haven't observed that in *Vicia* with th fractionated dose experiment. We kee our half doses low so that when we gi the combined treatments the yield of abe rations increases as the square of the dos

Abrahamson has noted that, althoug we do not see any significant difference after a single dose followed by treatmen with an inhibitor, there is perhaps a sligh increase. In Beatty's system, where h uses higher doses, he has been able to g after a single dose marked increases.

I don't think there is much movemen going on in the cell and John Evan work and Revell's work fits in very nicel with this point of view. However, ther might be some slight movement so th ends do move apart and reach anothe place where an interchange could occu to increase the numbers of visible aberr tions. Without this movement only rest tution could occur.

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[12] Peter Alexander, Chester Beatty Resear Institute.

[13] L. H. Gray, Mt. Vernon Hospital, Northwoo

RAY: The chances of moving apart
to me to be exactly balanced by the
ces of moving closer together.

OLFF: If the ends are close together
art with, I should think any movement
he average would tend to separate
. Then, since there are only a few
where breaks are within the rejoining
nce, such separation could only cause
loss of but a few exchanges. There
however, many more primary breaks
ent that were not within the exchange
nce of another break. If chromosomal
ement occurs that decreases restitu-
it seems to me that such movement
ld tend to increase the number of
anges by bringing distant ends closer
ne another. The original asymmetry
e distribution of breaks at sites versus
e not at sites would then favor an in-
se in exchange formation.

WANSON: Of course, at the end of a
sion there is an organized position of
mosomes with the centromeres at the
s and the ends dangling down, all of
ch might contribute to a higher fre-
ncy of breaks or a higher frequency of
ions. There is a factor of proximity
comes in which makes for breaks or
ions in the neighborhood of the cen-
ere much more likely than out at the
s, where they are dangling free. The
mosomes do not change position in the
leus very much until they begin to
ten, as was shown by Boveri.

VANS[14]: You can also show, in fact,
you get more association between
mosomes in the system Dr. Wolff is
ing about at the mid-chromosome re-
s, because that is where the hetero-
matin is located. The heterochromatin
s up in interphase to give the chromo-
ters, and a high aberration frequency
rs in these regions. In addition you
have the centromere suppressing aber-
on. There is a reduced frequency not
at the distal end of the chromosomes
also near the centromere.

WANSON: There is a problem when
is trying to compare *Tradescantia* and
a in the sense that we know next to
ing about heterochromatin in *Trade-
tia*.

VANS: No, but you have another in-
sting point there. In *Tradescantia*, I think you found reduced aberration frequencies at the chromosome ends.

SWANSON: This was in the pollen tube.

EVANS: The interesting point is that the nucleoli are organized at the distal ends.

SWANSON: Yes, on two of the 6 chromosomes.

EVANS: In our *Tradescantia* clone we have 6, one on each of the chromosomes and all located in a terminal position. Nucleoli certainly influence aberration yield by suppressing interchange in the nucleolar regions. The point I think we ought to make is that it isn't simply a question of how the chromosomes are arranged on the spindle; what is more important is their relation in interphase at the time they receive their radiation dose. We know that in the interphase nucleus in *Vicia* the two extremely large nucleoli must reduce the probability of close association between the nucleolar and other chromosome arms. On the other hand, the fused heterochromatic regions must enhance the possibility of exchange in and around the heterochromatic zones. This is especially important if, as Wolff points out, exchange only occurs at chromosome regions which are separated by very small distances, 0.1 of a micron or less.

SWANSON: This would account for it in *Tradescantia* just as it would in *Vicia*, but the circumstances are different. There is a different distribution present.

WOLFF: Let me say I think Dr. Evans is a bit modest here. From our data, our analysis is such that we couldn't do any more than define the sites as being the place where, by random association, the breaks happen to be within h microns of one another.

Evans has data that haven't appeared yet, but are in press, that indicate that the limited number of sites may perhaps have more meaning than just this random association; and indeed he is finding more exchanges in these heterochromatic regions than you would expect to appear on a random basis.

There are a small number of these heterochromatic regions, too.

EVANS: Can I just add to that? We found that we get a non-random distribution of interchanges and that our number of sites looks more likely to be two rather than the 4 that Wolff gets in *Tradescantia*.

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[14] H. J. Evans, Brookhaven National Laboratory.

WOLFF: We get two for *Vicia* also.

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stirradiation Processes in the Induction of Recessive hals by Ionizing Radiation

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the preceding paper, Dr. Wolff has ussed some of the postirradiation phe- ena that are important in the produc- of chromosome aberrations. Postirra- ion phenomena also occur for many of ne-hit events manifested as mutations review see Hollaender and Kimball, Conger, '60) as was first shown by ing that a variety of postirradiation tments modify the amount of muta- . Unfortunately, further exploration of phenomenon has been hampered by the of a conclusive test, comparable to the -fractionation test for two-hit events, the amount of recoverable damage re- ning at any moment after irradiation. way of attacking the problem is to t for varying times with agents whose hemical action is fairly well known to draw conclusions about the inter- iate steps from the results (Witkin, '58; Doudney and Haas, '58, '60). For *amecium,* at least, we believed that this hod was not very satisfactory because the many unknown side effects such nts might have on processes quite pe- eral to the mutation process itself. The st obvious peripheral effect is a change he time of the terminal event for muta- esis, which most of us now agree is A synthesis, although it is not difficult hink of other possibilities.

more direct measure of the rate of nge of reparable damage can be ob- ed by measuring the variation of muta- with the time between irradiation and terminal event. In *Paramecium,* this eriment is relatively easy because we work with small, synchronized groups ells and because the mutagenic doses ionizing radiation do not have appre- le effects on the time of DNA synthesis. use of this procedure, we have come to conclusion that the postirradiation phenomenon, in the case of one-hit recessive lethals, is one of metabolic repair of chromosomal lesions, analogous to the metabolic repair that occurs in restitutional reunion of broken chromosomes. It should be emphasized that there is evidence that recessive lethal mutations in *Paramecium* are one-hit phenomena and may be entirely localized changes such as point mutations and small deletions.

The methods of detecting and measuring mutation are given in earlier papers (Kimball *et al.*, '59a, b). Unless otherwise stated, X rays were used.

Repair during G1

Several years ago Kimball ('57) and Kimball *et al.*, ('57) showed that a variety of agents, streptomycin, chloramphenicol, H_2O_2, low temperature, starvation, caffeine, dinitrophenol, and iodoacetate, decreased mutation when used as postirradiation treatments. Various artifacts of selection and expression were excluded and on this basis it was concluded that the effect was on mutation itself. Similar phenomena were found with ultraviolet and alpha-particle irradiation (Kimball *et al.*, '59a). Further work has shown that modification can be brought about only before the first postirradiation chromosome duplication.

The successful treatments had only one thing in common as far as could be seen. They all delayed division, and those that have been tested also delayed DNA synthesis. This delay led us to postulate that the treatments decreased mutation by delaying chromosome duplication, thus allowing more time for repair of the initial premutational damage.

To test this hypothesis, we turned to experiments in which the time between

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[1] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

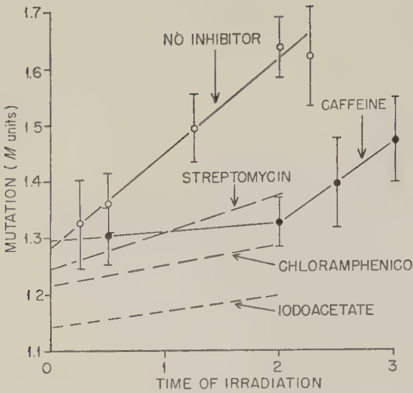

Fig. 1 Effect of time of irradiation on the amount of mutation. The data for no inhibitor and for caffeine (1.5 mM) include both previously published and new data and are shown in detail with 95% confidence limits. Lines of best fit to previously published (Kimball *et al.*, '61) data for growth in streptomycin (200 μg/ml), chloramphenicol (60 μg/ml) and iodoacetate (1.5 mM) are shown for comparison. The units in which mutation is given are discussed by Kimball *et al.* ('61).

irradiation and chromosome duplication was varied. The most direct evidence comes from experiments with stationary-phase paramecia (Kimball *et al.*, '59b). The time between irradiation and duplication was changed by varying the time of transfer to fresh culture medium, duplication occurring, of course, only after transfer. As expected on the spontaneous repair hypothesis, it was found that the longer the time between irradiation and transfer the less the mutation. That this is the result of repair after irradiation and not simply the result of the additional period under stationary phase conditions was shown by finding that the time in stationary phase before irradiation was unimportant.

Similar evidence was found with synchronized groups obtained by collecting dividing individuals from log-phase culture. Measurements of the size of the micronucleus and amount of Feulgen stain in such groups have shown that the micronuclear preduplication interphase (G1) lasts for about 2½ hours and the period of DNA synthesis (S) about half an hour; the remainder of the 5- to 6-hour total interdivision interval is occupied by postduplication interphase (G2) and mitosis (diagram at bottom of fig. 2). The ti between irradiation and duplication c therefore be varied from about 2½ to hours by irradiating at various times du ing G1. Fortunately the 4500 r of X ra used for most mutation experiments h almost no effect on the time of duplicatio

The combined results of a number of periments are shown in the curve mark "no inhibitor" in figure 1. It is clear th the longer the time between irradiation a duplication, the less the mutation. Th these experiments support the hypothe of spontaneous repair.

Experiments in which the time betwe division and irradiation is altered are su- ject to the alternative interpretation th the variation in mutation is the con quence of changes in sensitivity to t initial radiation damage. The repair h pothesis predicts the direction of chan that was actually found whereas the sta sensitivity does not. In addition, the da for stationary phase paramecia cannot explained by changes in initial sensitivi

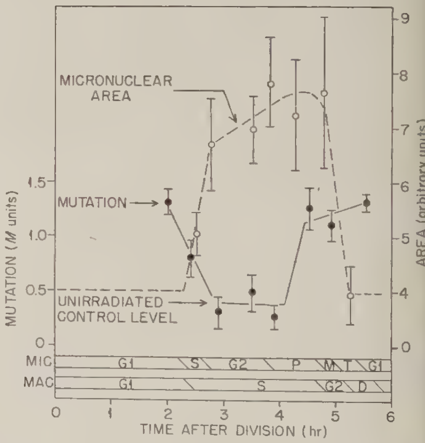

Fig. 2 Duration of the refractory period. M tation as a function of time of irradiation is co pared with data for micronuclear cross section area from the same experiment. Diagram at bo tom shows the approximate times occupied successive micronuclear and macronuclear stage The micronuclear data are from this same e periment; the macronuclear data are from earli quantitative Feulgen (Kimball and Barka, '59) a thymidine incorporation (unpublished) studie G1, preduplication interphase; S, period of DN synthesis; G2, postduplication interphase; P, pr phase; M, metaphase and anaphase; T, telophas D, period of amitotic division of the macronucle

all groups were irradiated together
iffered only in the time between irra-
on and transfer to fresh medium.
over there is some evidence (Kimball
, '61) from experiments in which the
of irradiation was held constant but
ime of posttreatment was varied that
is a loss of mutational potential dur-
og-phase growth. This question is dis-
ed at some length by Kimball *et al.*
). Thus the "stage sensitivity" alterna-
probably is not correct but cannot be
ously excluded.

he curve marked "no inhibitor" in fig-
suggests, in addition, that the rate of
ir is under metabolic control because
lope is about 10 times greater than
corresponding measure of the rate of
ir in stationary phase paramecia. A
nt of the log phase experiment was
to study further the role of metab-
n. Concentrations of several inhibitors
found that delayed but did not pre-
chromosome duplication and cell divi-
Cells were placed in the inhibitor
ediately after division and irradiated,
in the inhibitor, at appropriate times.
were not removed from the inhibitor
after the mutation process was
ght to completion by chromosome du-
tion. The results are diagrammed in
re 1 from more detailed data published
where (Kimball *et al.*, '61). All the in-
tors decrease the average rate of repair
hown by the slope of the lines. With
eine at least, the decrease is greatest
ng early G1 and becomes small or neg-
le in late G1. The change in slope of
curve is not to be explained by a lower
t for repair since considerably lower
ls of mutation are found with higher
centrations of the inhibitors (Kimball
l., '61). Thus these experiments sup-
the view that repair is a metabolic
cess that can be inhibited. They also
w that the consequences of such inhibi-
are dependent upon the stage during
interdivision interval. The dependence
stage and the similar action of diverse
bitors suggests that we are still some
ance from the specific biochemical re-
ons involved.

n apparent paradox exists in that the
e metabolic inhibitors that decrease
the rate of repair also decrease the amount of mutation when they are used as postirradiation treatments. Marked posttreatment effects are obtained only with considerably higher concentrations of the inhibitors than those in figure 1, but this does not resolve the paradox. It can be resolved, however, if it is recalled that the inhibitors also delay chromosome duplication, thus making more time available for recovery. The net result of these opposing actions is usually a decrease in mutation though there is no *a priori* reason why this should always be true. Indeed, the low concentrations of inhibitors which were shown in figure 1 to markedly decrease the rate of repair have almost no influence on the final number of mutations when used as posttreatments. Thus in *Paramecium* the effect of a posttreatment on the amount of mutation gives us no clue to the magnitude or even direction of the action on repair. Except in special cases, knowledge of the action on repair can come only from experiments in which the time between irradiation and chromosome duplication is varied.

Repair during G2

Thus far we have discussed only effects on the unduplicated chromosomes of stationary phase and of the G1 period of log phase. Irradiation of the duplicated chromosomes of G2 and early prophase produces no more than one-tenth the amount of mutation produced by irradiation at other stages. This refractoriness to mutation induction is found for alpha particles and 2537 Å UV as well as for X rays (table 1). The initial events with these three radiations are unlikely to be suffi-

TABLE 1

Amount of mutation by irradiation at various times after division

Time of irradiation (hours after division)	X rays	α Particles (Pu^{239})	Ultraviolet (2537 Å)
1	1.39 ± 0.08	0.68 ± 0.12	1.23 ± 0.13
2	1.48 ± 0.06	—	1.68 ± 0.10
3	1.22 ± 0.03	—	1.50 ± 0.11
4	0.07 ± 0.04	0.10 ± 0.05	0.30 ± 0.07
5	0.66 ± 0.03	—	0.16 ± 0.05

ciently alike to explain this similarity. The damage produced in G1 by all three radiations, however, has been shown to be subject to postirradiation repair (Kimball *et al.*, '59a). Therefore, we concluded that the refractory period is the result of postirradiation processes that remove all or nearly all the initial radiation damage produced during this period.

In normal, log-phase growth, the refractory period is coextensive with micronuclear G2 and early prophase but is also coextensive with the much longer macronuclear S period (fig. 2). Experiments with caffeine show, however, that the essential association is with the micronuclear and not the macronuclear state. Growth in 1.5 mM caffeine delays micronuclear G2 by about two hours and similarly delays the onset of the refractory period (fig. 3). Caffeine does not delay the onset of the macronuclear S period, although it slows down the rate of synthesis, as judged from the amount of tritiated thymidine incorporated during brief labeling periods (fig. 4). Thus the association is with micronuclear G2, not macronuclear S.

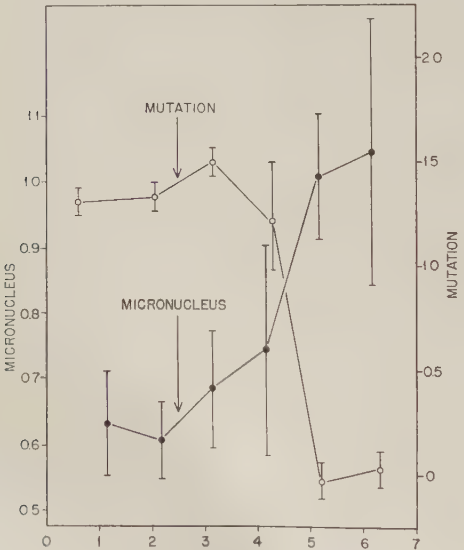

Fig. 3 Similar delay in the refractory period (from approximately 2½ to 4½ hours) and in micronuclear S by 1.5 mM caffeine. Micronuclear S determined by measurements of cross sectional area.

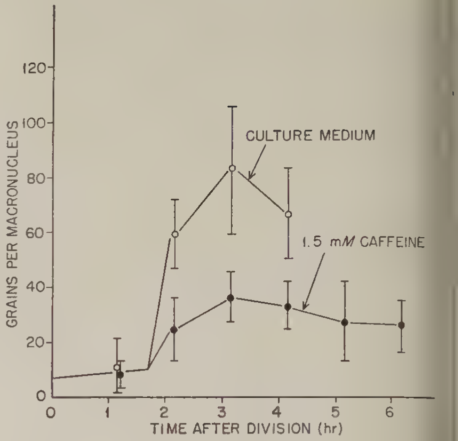

Fig. 4 Lack of an effect of 1.5 mM caffei
on the onset of macronuclear S but decrease
rate of synthesis. Measured autoradiographica
by the number of grains over a standard area
the macronucleus after a brief exposure to
thymidine.

Evidence that the refractory period is
consequence of the state of the micron
cleus and not of the whole cell is show
by use of the intracellular symbiont, kapp
Kappa is present in a number of strai
of *Paramecium* and can be inactivated
X rays (Preer, '48). We now find that it
equally inactivated whether the paramec
are irradiated in micronuclear G1 or
(34% survival of kappa in G1, 33%
G2). Inactivation of kappa, like mutatic
induction in the micronucleus, is infl
enced by oxygen tension during irradiatic
(Geckler and Kimball, '53). Therefore
would expect diffusible intracellular pr
tective agents and, with less certaint
other general conditions to influence bot
kappa and the micronucleus. The absenc
of a refractory period for kappa suggest
therefore, that the refractory period f
mutation induction results from local co
ditions within the micronucleus.

One other possibility that needed to
considered—although for several reaso
we thought it unlikely—was that the mut
tions examined were produced mainly
alterations of nucleoside or nucleotide pr
cursors of DNA. If this were the case, th
altered precursors might be retained in a
intracellular pool during G1 and also du
ing stationary phase but might be rapid
eliminated in prophase. Thus precurso
altered during G2 or early prophase wou

TABLE 2

tion by 5-bromodeoxyuridine at various
stages of the interdivision interval

e - rs r on)	No. of treated Paramecia	Amount of mutation	
		M	95% confidence limits
osure	194	0.25	0.30; 0.21
.4	80	0.25	0.32; 0.18
.7	194	0.59	0.67; 0.51
.5	80	0.32	0.41; 0.23

t before the next chromosome dupli-
and mutations could only be pro-
by irradiation in G1 or late mitosis.
absence of any information about
ypothetical altered precursors, it was
ed to try a model experiment with
ase analogue, 5-bromodeoxyuridine,
is known to be incorporated into
and to be mutagenic in microorgan-
(see, for example, Litman and Par-
60). As table 2 shows, this substance
tagenic for *Paramecium* when pres-
ring the period of micronuclear chro-
ne duplication but not when present
during early G1, G2, or early pro-
. Thus not enough of the analogue
etained in the cell from early G1 to
ation to produce detectable muta-
although a detectable effect would
been expected if this analogue had
behaving in the way postulated for
ypothetical altered precursors. It is
uded from this finding and *a priori*
derations that the altered precursor
hesis is improbable.

ere are two alternatives for explain-
he mutation curve in figure 2, espe-
the sharp break in late prophase, on
air hypothesis. (1) Premutational
ge is repaired very efficiently during
nd early prophase, possibly associated
doubleness of chromosomes, but is
red at the G1 rate from late prophase
(2) Repair is not necessarily very
ent during G2 and early prophase but
remely efficient during late prophase
at any damage produced earlier is
nated before the next chromosome
cation. We can see no good basis for
e between these alternatives at pres-

Our results with G2 irradiations in *Paramecium* and those with plant chromosomes stand in contrast to one another. As Dr. Wolff pointed out in the preceding paper, the proximity of the sister chromatids favors unions between neighboring breaks, resulting in more detectable damage. Such isochromatid sister unions should act as recessive lethals in the Paramecium detection system, but the low frequency of mutations during G2 suggests that they do not occur. This is in line with other evidence (Kimball *et al.*, '57; Wells, '60) that gross chromosome aberrations are rarely if ever induced in ciliates by even quite high doses of radiation.

Whatever the nature of the mutations, the very small number produced during G2 is strong evidence that localized chromosome lesions can be repaired. It is nearly inconceivable that doses of several thousand roentgens would produce only the small number of molecular alterations in the chromosome that are observed as final mutation. The only other possibility is that the original alterations are to a large extent reparable.

CONCLUSIONS

Studies on recessive lethal mutations in *Paramecium* suggest at least two postirradiation processes: (1) loss of premutational damage by what can best be looked upon as metabolic repair of localized chromosome lesions and (2) conversion of remaining damage to mutation at the time of DNA synthesis. Little if any mutation can be produced by irradiation in G2 and early prophase, suggesting that repair of chromosome lesions is remarkably efficient during one or both of these periods.

OPEN DISCUSSION

Swanson[2] : A problem the future holds for us probably is a redefinition of a lesion. We have called it many things—metastable state, active state, potential breaks—but we still wonder what we mean in many instances. The subject is open for discussion.

Burdette[3] : Mr. Chairman, if you will permit extending this discussion to the metazoans before the next session, I would like to show briefly some recent results obtained in our laboratory concerning the protective role of antibiotics on lethal mutation rate in *Drosophila* following ionizing irradiation.

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[2] Carl P. Swanson, The Johns Hopkins University.

[3] Walter J. Burdette, University of Utah.

Both actinomycin D and penicillin were tested by adding them to the medium and then irradiating males with 3000 r (6 ma, 100 kvp, 1.0 mm Al, 15 cm). Lethal-mutation frequency on the X chromosome was determined by utilizing the Muller-5 method by which it is possible to preserve and retest each lethal in subsequent generations. Ordinarily only two or three lethals are encountered among 1000 X chromosomes tested.

In one set of experiments, mutation frequency was raised to 5.73% (67/1168) with irradiation, but this was reduced to 3.25% (31/952) when actinomycin D was added to the medium before irradiation. When no X irradiation was used, the rate was 0.10% (1/978) with actinomycin D treatment and 0.14% (2/1375) in the control group. In the irradiated group, percentage mutation rate was reduced from 6.77 (68/1004) to 3.76 (39/1036) when penicillin was substituted for actinomycin D in the medium. Eight lethals were found among 1150 chromosomes tested when this antibiotic was used without irradiation. Sufficient data are not at hand to resolve the interesting speculation that penicillin may enhance the frequency of natural mutations.

In a series of experiments in which the effect of penicillin was compared to that of N_2O, the rate of lethals following irradiation alone was 4.67 (67/1434); with irradiation and penicillin it was 2.64 (36/1361); and with irradiation in the presence of N_2O it was 1.15 (18/1555). Penicillin added to treatment with N_2O did not depress mutation rate additionally when compared to the rate of N_2O and irradiation, but the mutation rate of 1.17 (21/1789) was less than the values for irradiation and penicillin.

When the rate of lethal mutations following irradiation was determined for various stages of spermatogenesis from spermatozoa to spermatogonia, the lower rate with penicillin was found at each stage.
This was also true when N_2O was added
the irradiation.

It seems justifiable, then, to conclu
that both antibiotics studied cause a
spectable and significant alteration in
frequency of lethal mutations after irrad
tion in *Drosophila*. Further, this consis
of a uniform diminution of rate through
spermatogenesis, and quantitatively
phenomenon differs from the reduction
mutation frequency induced by the g
N_2O.

DOUDNEY[4]: I would like to descr
some of the studies of Dr. Kada, in
group, which suggest that the chlora
phenicol effect might hold for X-ray as w
as UV-induced mutation.

Dr. Kada irradiated *E. coli* WP2,
tryptophan-requiring strain, with about
kr of X rays and incubated subsequen
in liquid medium containing a rich supp
of amino acids including tryptophan. T
plating media he used were both nutrie
broth and minimal agar medium not c
taining any nutrient broth. Thus, in
case he was measuring what we assume
be the mutagenic event, as I describ
earlier in my paper and, in the other ca
he was measuring the expression of t
mutation in the same way in which
were studying it with UV.

When he added chloramphenicol i
mediately after irradiation, incubated
increasing periods of time, and plated
minimal agar, he got a very low level
mutation frequency. Thus, it is clear th
with incubation no mutations are
pressed in the presence of chlorampheni
icol. If, however, he plated on nutrie
agar containing medium he got 100%
the mutations back. Thus, X-ray mut
tions are not sensitive to chloramphenic
in the same way that the UV-induc
mutations are. If he waited 10 minut
after irradiation before he added chlora
phenicol, he found a typical mutation fr
quency decline process which is very co
parable to what we get with U
Approximately 50% of the X-ray-induc
mutations proved to be sensitive to chl
amphenicol added after 10 minutes.

The other 50% of the mutations we
being expressed, as measured by plati

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[4] C. O. Doudney, The University of Texas, M.
Anderson Hospital.

inimal medium, during this same
l, so that after approximately 10 min-
incubation, he found that approxi-
y half of the mutations were al-
expressed as measured by plating on
nal medium and the other half were
ive to chloramphenicol. This ac-
ed for all the mutations.

da then turned to studies of a thy-
less, amino acidless strain. He fol-
reversion of the amino acid require-
in the same way both with and
ut thymine. He found that approxi-
ly half of the mutations were ex-
ed in the absence of thymine in the
20 minutes as measured by plating
quently on minimal medium contain-
hymine but not the amino acid. Ex-
ion then levels off at around 50% but
he added thymine before plating, he
he other 50% back. It thus appears
there are two classes of mutations
X-ray exposure. One half of the
tions requires no thymine and prob-
no DNA synthesis for induction. Ex-
ion occurs very rapidly within 10 min-
after exposure. This half may be due
direct effect on the DNA. The other
of the X ray-induced mutations are
tive to chloramphenicol and require
synthesis for induction and subse-
t expression.

RAHAMSON[5] : Would Dr. Kimball com-
on why isochromatid breaks would
to lethals?

MBALL: An isochromatid break will
uce a terminal loss of chromosome
rial and when such deficient chromo-
s are made homozygous at autogamy
will act as lethals. There is reason to
ve that there is no selective elimina-
of either nuclei or whole cells before
gamy, therefore such deficient nuclei,
oduced, should survive long enough to
rise to lethal segregants at autogamy.
ve can say is that such segregants do
occur after doses of about 5 kr of X
given in micronuclear G2.

ELLY[6] : Dr. Kimball, you mentioned
5000 r in G1 does not delay the entry
S. Have you published this?

MBALL: We have found (Kimball,
ler, and Gaither, J. Cellular Comp.
iol., *40:* 427, 1952) that doses of
t 5 kr of X rays delay division only
about 10 minutes. Other agents (caffeine, chloramphenicol, 70 kr of X rays) delay division and micronuclear division equally. Thus, it seems reasonable to conclude from the small delay in division that there is no more than a small delay in micronuclear duplication. We intend to check this point more carefully.

SWANSON: Are you worried that this organism can take this kind of radiation and still plug along? It can withstand a tremendous amount, of course.

KELLY: I know. But this is a question of the sensitivity of a biochemical system. It is currently said any cell, once it is in S, is able to continue DNA synthesis; but that the biochemical lesion is one in G1 which prevents the entry into synthesis.

KIMBALL: At considerably higher doses, we delay division and both macronuclear and micronuclear S by irradiating in G1. The system is very resistant but not completely so.

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[5] Seymour Abrahamson, University of Wisconsin.

[6] Lola S. Kelly, University of California, Berkeley.

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Round Table Discussion

AIRMAN CHARLOTTE AUERBACH[1]: Last
I felt depressed about this evening
ng because we had heard so much
the wonderfully predictable ways in
dry spores and free DNA behave;
felt we were coming on to something
edingly different tonight when we deal
living metazoans.
t today we have had a transition to it.
ave had already good examples of
much more complicated living cells
han dried spores or even dried seeds
e free transforming principle. In to-
t's discussion we are going even a step
er because we are not going to talk
t the recovery of individual cells but
ll populations; and a difficulty which
hall have to discuss is that selection
een cells may lead to results which
to be distinguished from recovery.
fore I introduce the speakers and our
, I should like to mention that there
e class of recovery effects in germ
with which we are not going to deal
use this work has already been pub-
d; but it may not be known to every-
that recovery from UV radiation, both
gard to killing and in regard to muta-
was found in *Drosophila* almost at
ame time, or immediately after it had
found by Kelner in bacteria.
night we shall deal exclusively with
appears to be, or may be—it may
out in the discussion—recovery from
y effects, both in respect to the killing
rm cells and in respect to mutation.
have two people dealing with mouse
, two with *Drosophila* work; and I
have a paper on silk worms which was given to me in Japan on which I shall report briefly.

The main questions which we are going to discuss are these: First of all, dose-rate effects will be described and we shall discuss whether or not they are due to recovery. Then, secondly, we shall hear about the effect of fractionation. Thirdly, I shall report briefly some data by Dr. Sobels, who unfortunately couldn't come. His subject is posttreatment after irradiation.

Finally we shall come to effects which are specific to *Drosophila*. When *Drosophila* males are irradiated and mated on the first day and then again on the second day—or only on the second day—there is a difference in mutation frequency between the first and the second day—mutation frequency is lower on the second. We shall hear new data about this from Dr. Lüning, and we shall have to discuss whether this is a recovery phenomenon.

Another specifically *Drosophila* phenomenon which in my opinion very probably is related to the previous one is this: when *Drosophila* spermatozoa are irradiated in inseminated females, higher mutation frequencies are obtained than when they are irradiated in the males. New data on this point will be presented by Dr. Oster, who tested for such a difference after treatment with neutrons, which do not give the first *versus* second day effect.

We shall start with Dr. Oakberg.

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[1] Charlotte Auerbach, Institute of Animal Genetics, Edinburgh.

ect of Dose and Dose Rate on Radiation Damage to ouse Spermatogonia and Oocytes as easured by Cell Survival

E. F. OAKBERG AND EVELYN CLARK
Biology Division, Oak Ridge National Laboratory,[1] Oak Ridge, Tennessee

Ve have studied the effect of low total es and of low-dose rates on spermato- ia and oocytes of the mouse with X- l γ-ray irradiation. Neutron exposures ow doses were used for relative biologi- effectiveness (RBE) comparisons with ays. The same basic problems may be olved with both low dose rates over long e periods and with small total doses at her dose rates, since both procedures ult in reduction of the number of ioni- ions absorbed per sensitive target vol- e per unit time. These results, there- e, are of interest not only in demon- ating the effect of small radiation doses, also in evaluating the importance of turbances in cell dynamics in the de- ndence of mutation frequency on dose e.

Interest in the effect of varying the time which a radiation dose is administered veloped early in the history of radiobiol- y. Total exposure time can be varied by anging the dose rate, by dose-fractiona- n with rest periods between exposures, by various combinations of these proce- res. As early as 1911, Regaud and gier, who were studying the effects of rays on the ram testis, concluded that ctionation was more efficient than a gle exposure in the induction of steril- . More recently, however, Kirchhoff and lbling ('37) observed no difference in ect on the rabbit testis when the same al dose was divided into 6–10 daily frac- ns, or given in one acute exposure. Kohn d Kallman ('55) concluded that there s no fractionation effect on the mouse tis if the doses were given within a 4- y interval. If the individual dose frac- ns are small enough, however, as the r per day used by Langendorff and ngendorff ('57), the biological effect is reduced, and some male mice remain fertile after total doses of 400 to 800 r. In a quantitative study of cell populations in the mouse testis, Eschenbrenner *et al.* ('48) observed a similar reduction of effect with chronic irradiation at greatly reduced dose rates. They concluded that equilibria were established between destructive actions of the radiation and regenerative capacity of the seminiferous epithelium, with dose rate, not total dose, being the primary determining factor. Thus evidence for reduced effect on the seminiferous epithelium has been demonstrated both for doses divided into small fractions (Langendorff and Langendorff, '57) and after continuous irradiation at low dose rates (Eschenbrenner *et al.*, '48). It is not clear, however, whether this response is the result of improved survival of individual cells, or whether it arises from compensatory alteration in dynamics of the spermatogonial population. Furthermore, these apparent differences between experiments in the effect of fractionation must be evaluated with the realization that synchrony of treatment with the dynamics of spermatogenesis may not have been the same in all these experiments, especially where different species were used.

Radiation damage at the cellular level is more clear-cut in the female since a fixed population of oocytes, that cannot be replenished in the adult, is available for treatment and analysis. Fertility is greatly improved with fractionation or continuous irradiation at low dose rates (Russell *et al.*, '59), a result which can be due only to improved survival of individual cells.

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[1] Operated by the Union Carbide Corporation for the U. S. Atomic Energy Commission.

Recently, Russell and Kelly ('60) and Russell (companion paper, this volume) report a frequency of mutations from spermatogonia irradiated at 0.8 r/minute that is comparable to the frequency observed at a dose rate of 0.009 r/minute, but which is significantly below the rate obtained at 90 r/minute. The animals irradiated at 0.8 r/minute regained fertility at about the same time as those irradiated at 90 r/minute, suggesting that, while mutation frequency changed over this range of dose rate, killing of spermatogonia was unaffected. While some significant effects of dose rate on cell survival have been observed in the present experiments, the differences are small, and would not be expected to affect the time at which fertility was regained by irradiated males. Cell survival per roentgen is enhanced by dose rates lower than those cited above and by small acute doses. Similar results have been observed in females, suggesting common factors in radiation response of both spermatogonia and oocytes.

MATERIALS AND METHODS

Males. Male F_1 hybrid mice, from the cross of inbred 101 females with C3H males were 12 weeks old at the time of irradiation. X-ray exposures were made with a GE Maximar operated at 250 kv, HVL 0.4 mm Cu, inherent filtration 3 mm Al, 15 ma for the 86 r/minute and at 1.5 ma for the 9.3 r/minute dose rates. Cs^{137} sources were used for γ-ray exposures at 0.8, 0.009 and 0.001 r/minute. Co^{60} γ rays, at 3.8 r/minute, were used for low acute doses in the γ ray-neutron comparisons. RBE determinations were based on irradiation with neutrons of average energies of 2.5 and 14.1 Mev from a Cockcroft-Walton linear accelerator.

All males were killed by cervical dislocation 72 hours after exposure and the testes fixed in Zenker-formol. Five-micron paraffin sections were stained by the PAS technique, with Ehrlich's hematoxylin as a nuclear stain. Ninety-nine tubule cross-sections, distributed among the different stages of the cycle on the basis of a previously determined frequency of tubule stages (Oakberg, '56) in control mice, were scored for each mouse. Round tubules, with regular cell layers, were selected under low power, and spermatogonial cou
were then made using the oil immers
lens. Preleptotene spermatocytes in st
VII tubules were also counted. All cou
were converted to experimental/cont
ratios.

Females. Hybrid (101 × C3H) fema
were irradiated when 3–4 months old.
rays, 86 r/minute, were used for the 5
exposure. A Co^{60} source giving a dose r
of 2.85 r/minute was used to give do
of 1–25 r. Continuous irradiation at 0.0
r/minute was obtained with a 5 Curie C
source. At 0.009 r/minute the expos
times were long, and the onset of radiati
was timed in such a manner that the
males were of the same age (±3 days)
the completion of exposure. Mice were ra
domized among the various dose grou
and the unirradiated animals were h
until completion of irradiation in order
control the decrease in oocyte numb
known to occur with increase in age.

All mice were killed 72 hours after
radiation, and the ovaries fixed in Zenk
formol. Seven micron serial sections we
prepared and stained in hematoxylin Azu
II-eosin. The survival curves in figure

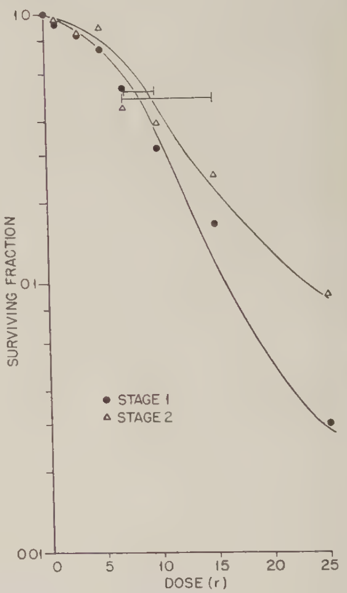

Fig. 1 Survival of stage I and stage II oocy
of the 10-day mouse 72 hours after exposur
Co^{60} γ rays. Indicated LD_{50}'s for stage I are 8
(95% confidence limits 7.2 and 9.7 r) for stage
9.4 r (95% confidence limits 6.7 and 14.
respectively.

e based on the number of oocytes in 8 s sections from the central portion of series for each ovary. Adjacent sec- s and imperfect sections were not used. a in table 4 are based on counts of the re series for each ovary. Data pre- ted here refer only to stage I and stage ocytes; i.e., oocytes with only a few as- ated follicle cells or a single layer of ened cells, respectively.

RESULTS

ales. The square root of the cell count used for statistical analysis of the ct of dose rate on survival of type A rmatogonia. Variance analyses were on the 11, 21, and 100 r doses at 86, and 0.8 r/minute as a group, and on 5 and 11 r exposures at 0.8 r/minute 0.009 r/minute as a group. A com- ison of the three dose rates was also de within each of the 300 and 600 r osures for each killing time. Signifi- t dose-rate differences were observed the 11–100 r (F = 9.66, df 2 and 54) 300 r 5-day (F = 7.86, df 2 and 18) ups.

hree and one-half minutes and 6 hours 33 minutes are required to give 300 r 86 r/minute and at 0.8 r/minute re- ctively. On the other hand, it takes t over 23 days to administer 300 r at 09 r/minute. Thus, while it is conceiv- e that the dynamics of spermatogonial wth and division do not complicate the ult in the 3½ minute vs. 6½ hour com- rison, they would do so in the 6½ hour 23-day comparison. For this reason it s necessary to make some dose-rate comparisons at low total doses where it was possible to stay within a relatively short time interval even at the lowest dose rate.

At 5 and 11 r, the extent of cell killing was slightly lower than that observed at higher dose rates (table 1). This dose rate of 0.009 r/minute must permit some recovery at the cellular and/or population level, however, since survival, observed after 300 r, was significantly improved over that obtained after higher dose rates. (In order that the comparisons be made on minimum survival for all dose rates, the data for 0.009 r/minute were restricted to 6, 12, and 18 hours after the end of irradiation. At later intervals, spermatogonial numbers in chronically irradiated mice are significantly increased by cell division.) Eventually, however, the mechanism giving improved survival after 300 r at 0.009 r/minute breaks down, and the number of spermatogonia is 6% of control at 600 r, which is not significantly different from survival at higher dose rates. Type A spermatogonia were reduced to only 80% and 85% of control by exposure to 100 and 300 r respectively, at 0.001 r/minute. This repopulation indicates either greatly reduced cell-killing, significant alterations in the cycle of germ cell renewal to maintain cell populations, or differential killing of the various developmental stages of type A spermatogonia. At 0.001 r/minute, not even low total doses can be used to analyze cell-killing independently from cell repopulation, since the time required to give even 5 r greatly exceeds spermatogonial generation time.

TABLE 1

Experimental/control ratios for type A spermatogonia after irradiation at different dose rates

Dose	Post-irradiation interval	Dose rates, r/minute				
		X rays		Cs^{137} γ rays		
		86	9.3	0.8	0.009	0.001
r	*days*					
5	3			0.856	0.933	
11	3	0.810	0.825	0.758	0.783	
21	3	0.611	0.710	0.570		
100	3	0.220	0.260	0.190		0.796
300	3	0.119	0.134	0.152	0.372[1]	0.846[1]
300	5	0.075	0.083	0.104		
600	3	0.075	0.086	0.095	0.061[1]	
600	5	0.026	0.028	0.042		

[1] Combined values for 6, 12, 18 hours after end of irradiation.

TABLE 2

Experimental/control ratios for type A spermatogonia, type B spermatogonia, and preleptotene spermatocytes after gamma ray and neutron irradiations

Dose	Co^{60} γ rays				2.5 Mev neutrons				14.1 Mev neutrons		
	Experimental/control				Experimental/control				Experimental/control		
	A	B	RPS[1]		A	B	RPS[1]		A	B	RPS[1]
rad				*rad*				*rad*			
1	1.061	1.066	1.068	0.25	1.047	0.986	0.947	0.47	1.047	0.973	0.990
2	1.017	0.976	0.985	0.51	1.123	0.973	1.002	0.98	0.937	1.014	0.957
3	0.972	0.943	0.959	0.72	1.035	0.985	1.040	1.51	0.947	0.934	0.980
4	0.968	0.935	1.005	0.96	1.107	0.949	1.007	2.43	0.916	0.923	0.927
5	0.982	0.956	0.928	1.47	1.037	0.945	1.020	3.0	0.859	0.890	0.907
6	0.989	0.958	0.948	1.98	0.907	0.829	0.915	5.0	0.805	0.867	0.856
8	0.889	0.878	0.910	3.59	0.871	0.769	0.839	7.1	0.767	0.718	0.588
10	0.904	0.929	0.923	5.09	0.867	0.804	0.790	10.0	0.739	0.685	0.548
12	0.845	0.829	0.870	7.42	0.753	0.730	0.722	15.0	0.558	0.466	0.375
23	0.698	0.710	0.638	10.49	0.700	0.544	0.504	19.8	0.451	0.274	0.206
38	0.537	0.435	0.317					24.1	0.389	0.243	0.123
57	0.427	0.255	0.141					30.0	0.334	0.146	0.071
80	0.343	0.156	0.052								

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[1] RPS, preleptotene spermatocytes of stage VII tubules.

TABLE 3

Estimates of cell survival based on total[1] cell counts

	Co^{60} γ rays		2.5 Mev neutrons			14.1 Mev neutrons		
e	Total cells	Exp./Control	Dose	Total cells	Exp./Control	Dose	Total cells	Exp./Control
			rad			*rad*		
	9,086		0	7,572		0	7,572	
	8,071[2]	1.066	0.25	7,496	0.990	0.47	7,554	0.998
	7,498[2]	0.990	0.51	7,735	1.022	0.98	7,393	0.976
	8,687	0.956	0.72	7,690	1.016	1.51	7,210	0.952
	8,778	0.966	0.96	7,637	1.009	2.43	7,011	0.926
	8,672	0.954	1.47	7,521	0.993	3.0	6,723	0.888
	8,756	0.964	1.98	6,639	0.877	5.0	6,420	0.848
	8,101	0.892	3.59	6,197	0.818			
	8,370	0.921	5.09	6,183	0.817			
	7,690	0.846	7.42	5,556	0.734			
	6,222	0.685						

Counts for type A spermatogonia, type B spermatogonia, and resting primary spermatocytes com-
d.

Data based on 5 mice per dose for 1 and 2 r; 6 mice per dose for rest of γ ray exposures; 5 mice
dose for neutrons.

n order to obtain improved statistical
ability, counts for the three different
types (table 2), were combined over
range of doses where no significant
erences attributable to cell type oc-
red (Oakberg, '57). An effect was ob-
ved for 3 rads of Co^{60} γ rays, 2 rads of
Mev neutrons, and 1.5 rads of 14.1
v neutrons (table 3). The lack of re-
nse at lower doses may be explained
er by a multi-hit basis for cell killing,
by cellular repair and/or protection.
se results are in agreement with the
ults of Langendorff and Langendorff
7), who divided the total dose into 2.5 r
ctions. It should be pointed out, how-
r, that in the present material, the lack of response at low doses could result from technical difficulties. Differences in section thickness and intensity of staining could mask the effect of 1 or 2 rads.

The relative biological efficiencies of neutrons as compared to γ rays computed from the data of table 2 are given in table 4. Two sets of RBE's were computed, one for each cell type over the entire dose range for the two neutron energies, and a second set for the combined survival of all cell types at low doses. In the latter computation, data for the three cell types were combined for the dose range where no significant differences in survival of the different cells were observed, i.e., for 1–23 rad for Co^{60} γ rays, 0.5–5 rad for 14.1 Mev,

TABLE 4

RBE of neutrons to Co^{60} γ rays for spermatogonial killing

Neutron energy	Cell type scored	RBE		
		Lower 95% confidence limit	Point estimate	Upper 95% confidence limit
14.1 Mev	A	1.41	1.76	2.76
	B	2.19	2.52	2.89
	RPS	2.11	2.38	2.69
	Pooled[1]	4.39	8.18	16.42
2.5 Mev	A	1.26	1.89	3.32
	B	1.22	1.85	2.53
	RPS	0.73	1.10	1.49
	Pooled[1]	0.68	3.03	6.80

[1] All three cell types combined for *low* dose ranges only: (1–23 rad for Co^{60} γ rays, 0.5–5.0 rad for 14.1 Mev neutrons, 0.25–7.42 rad for 2.5 Mev neutrons).

TABLE 5

Effect of dose rate on survival of primary oocytes of the mouse

Age at exposure[1]	No. of mice	Dose	Radiation	r/minute	No. of cells	Experimental / Control
days		*r*				
10	1	0	—	—	4,192	
10	1	25	Co^{60} γ	2.85	82	0.030
10–12	1	0	—	—	5,090	
10–12	1	26	Cs^{137} γ	0.009	672	0.132
7–14	1	0	—	—	4,819	
7–14	1	91	Cs^{137} γ	0.009	2	0.0004
70	3	0	—	—	9,121	
59	3	50	X rays[2]	87	12	0.0013
137	1	0	—	—	1,303	
133–136	1	44	Cs^{137} γ	0.009	407	0.312
125–131	1	87	Cs^{137} γ	0.009	364	0.279
119–132	1	164	Cs^{137} γ	0.009	24	0.074

[1] All mice killed 72 hours after exposure.
[2] Head shielded with lead during irradiation.

and 0.25–7.42 rad for 2.5 Mev neutrons (table 3). These "pooled" RBE's (table 4) of 8.18 for the 14.1 Mev and 3.03 for the 2.5 Mev neutrons were higher than any values obtained for the separate cell types. This difference was significant for the comparison between "pooled" and all three cell types for 14.1 Mev, and for the "pooled" vs. type B and RPS comparisons for 2.5 Mev neutrons. The lower RBE with 2.5 Mev rather than with 14.1 Mev neutrons was unexpected, but the difference is not statistically significant. However, it may be compatible with the conclusion of Storer *et al.* ('57) on optimum energies for maximum RBE.

Females. Primary oocytes in early follicle stages provide unique material for evaluation of the effect of radiation at the cellular level, since (1) they are in a relative uniform meiotic prophase stage, the dictyate, (2) they are sensitive to radiation-induced cell death, and (3) their losses cannot be replaced by cell division. Reduction of the dose rate, in both young and adult mice (table 5) significantly decreases cell death. In young mice, only 3% survival of stage I oocytes was observed after 25 r of Co^{60} γ rays given at 2.85 r/minute, but 13% survival was obtained after 26 r of Cs^{137} γ rays at 0.009 r/minute. In adult mice, not even 164 r at 0.009 r/minute reduced oocyte numbers to the 0.13% survival observed after 50 r of X rays at 87 r/minute. Eighty-seven r at 0.009 r/minute gave 28% survival in adult females, which is higher than the
13% observed after 26 r to young mice
the same dose rate. Thus, we conclu
that either oocytes in young mice are m
sensitive to radiation, or that repair pr
esses are more efficient in adult female

Curves for cell survival at low ac
doses comparable to those observed
spermatogonia are obtained for stage I a
II oocytes (fig. 1). The shoulders on
curves occur below 10 r, just as for sp
matogonia. Although we have no co
parable dose curves for oocytes of ad
females, the similar response of oocytes
young and adult females to reduction
the dose rate suggests that adult fema
also should show a similar response at l
acute doses.

DISCUSSION

The mechanisms involved in radiati
induced cell death are not well understo
but probably are quite varied. The o
mechanism directly observed thus
is the heteroploidy and/or aneuploidy
daughter nuclei arising from irradiati
of the parent cell. This must be a mi
factor in these experiments, however, si
most spermatogonia probably die in l
interphase or early prophase before di
sion (Oakberg, '55), and early oocytes
in meiotic prophase (dictyate) and usua
die within hours after irradiation, bef
meiotic division is possible. While o
the nucleus has been shown to have se
tivity compatible with effects obser
after the doses used in these experime
the possibility of effects on other cell s
tems has not been excluded. Since red

of the dose rate to 0.009 r/minute im-
es survival at 300 r (table 1) and a
her reduction to 0.001 r/minute gives
arked increase in survival, some repair
adiation damage must be occurring.
n repair may possibly involve direct
ection by various systems of the cell,
could be a repair mechanism depend-
on cellular metabolism as has been
wn for chromosome rejoining (Wolff
Luippold, '55; Wolff, '60), and for
pletion of certain mutations in para-
ia (Kimball *et al.*, '61).
urvival curves suggest that multi-hit
nomena are involved both in the killing
ocytes, which are in a single meiotic
phase stage (dictyate), and of all three
s of spermatogonia, which are in vari-
stages of the mitotic cycle. There is
rea of less slope (shoulder) on the sur-
l curves below 10 r both for oocytes
. 1) and for spermatogonia (tables 2
3). It is especially important that
shoulder also is obtained with scoring
both preleptotene spermatocytes and
ytes, since the spermatogonia that give
to preleptotene spermatocytes in stage
tubules 72 hours after irradiation al-
dy have differentiated irreversibly to-
rd production of sperm, and oocytes are
neiotic prophase and cannot be replaced
mitosis. Thus the response in these
s is independent of the possibility of cell
lacement which could confound the
onse of type A spermatogonia. Since
cell types show this shoulder at low
es, the simplest hypothesis is one postu-
ng a similar mechanism for cell-killing
all spermatogonial and early oocyte
ges.

These changes in RBE with dose (table
could arise from different dose-response
ves, for example, a straight line for neu-
ns and a curvilinear one for γ rays
ld give higher RBE's at low doses. The
a (tables 2, 3), however, indicate that
basic shape of the curves is the same
γ rays, and for 14.1 Mev neutrons,
h a shoulder and a succeeding approxi-
tely linear component on a semi-log
t. 2.5 Mev neutrons, however, give an
ponential curve. Also, it might be ex-
cted that the higher LET of neutrons
uld result in irreparable damage to the
jority of those cells "hit", while with less densely ionizing γ rays the probability of accumulating enough multiple hits to give lethal damage would be smaller at low doses. In this context, it is puzzling that the lowest dose of neutrons did not give some effect, but show a range of doses with no effect just as for γ rays. This probably is not a true biological phenomenon, but the result of variability introduced by differences in section thickness and staining intensity. One must consider, also, that even with radiation of high LET there must be a surrounding zone of diminishing effect for each "track" in which distribution of ionizations may be comparable to those obtained with γ rays. Thus at least part of the reduction in effect with low neutron doses may be comparable to that observed with γ rays.

One of the most pertinent comparisons to be made with the data of table 1 is with mutations produced in type A spermatogonia by irradiation at 0.8 r/minute, where Russell and Kelly ('60) observed a frequency which agreed closely with the results after 0.009 r/minute, but was significantly lower than the frequency at 90 r/minute. The significant effect of dose rate on spermatogonial survival observed at 11, 21, and 100 r at 86, 9.3, and 0.8 r/minute can be attributed to a low value at 0.8 r/minute, an intermediate one at 9.3 r/minute, and the highest value at 86 r/minute. Therefore, while showing an effect, the change does not parallel change in dose rate (table 1), and it is possible that technical errors are responsible. This is even more likely in view of the fact that there is little or no dose-rate effect on survival at the higher doses, and if there is an effect, it is in the opposite direction from the above.

In three of the four comparisons at higher doses, the difference in survival was not significant at the 5% level. In the remaining one, 300 r 5-day group, the difference was significant at the 1% level. At the dose of 600 r as used in W. L. Russell's mutation study, survival was not significantly affected by changes in dose rates. This suggests that the mechanisms involved in mutation production may be different from those leading to cell death. Also, this result strongly supports the previous conclusion of Russell *et al.* ('58, '59) that the effect of dose rate on mutation frequency in spermatogonia is not the result of cell selection.

SUMMARY

1. While some significant differences attributable to dose rate were observed, they were not consistent throughout the experiment. In order of increased survival, the rank was 0.8, 86, and 9.3 r/minute for doses of 11–100 r, and 86, 9.3, and 0.8 r/minute at 300 and 600 r.

2. Russell (companion paper) observed a 4-fold reduction in mutation frequency over the 86 to 0.8 r/minute dose range at a total dose of 600 r. In the present experiments at these dose rates spermatogonial survival for 600 r is highest, but not significantly so, at 0.8 r/minute. Thus these data support the conclusion that cell selection is not involved in the dose-rate effect on mutation frequency in spermatogonia.

3. A marginal effect of dose rate on spermatogonial survival was observed at 0.009 r/minute, and significant improvement in survival was obtained at 0.001 r/minute.

4. Reduction in number of spermatogonia was detected after 3 rad of Co^{60} γ rays, 2 rad of 2.5 Mev neutrons, and 1.5 rad of 14.1 Mev neutrons. The lack of effect at lower doses probably is not of biological origin, but the result of variation introduced by technical difficulties.

5. Both oocytes and spermatogonia give multi-hit survival curves, with a shoulder in the 3–10 r dose range, i.e., doses high enough to give a detectable effect. Since this effect was obtained both for oocytes and for spermatogonial stages where no cell replacement by division could occur, phenomena at the cellular level are indicated.

6. Since any loss of oocytes cannot be replaced by cell division, the improved survival of these cells at 0.009 r/minute over higher dose rates conclusively demonstrates the existence of cellular systems which either prevent or repair a portion of the radiation damage. The fact that oocyte survival after chronic irradiation is higher in adult than in young females suggests either less effective elimination of radiation damage, or a higher sensitivity of
comparable oocyte stages in young
males.

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G. Gosslee for statistical analysis of
data.

OPEN DISCUSSION

Monesi[2] : In one cycle of the ser
niferous epithelium of the mouse testis
generations of type A spermatogonia, c
of intermediate and one of type B sperm
togonia occur, as shown by using tritiat
thymidine and autoradiography. The ge
eration time is about the same in all
generations, or 27 to 30 hours. The p
DNA synthetic time, including the mito
stages from metaphase through telopha
and the resting phase preceding DNA sy
thesis is also fairly uniform, or 8 to 10
hours. The DNA synthesis, that occu
throughout the second half or the last 4
of the interphase, and the post-DNA sy
thetic time, that corresponds essentially
the duration of prophase, are instead ve
different from one generation to anoth
DNA synthesis is very long, about 14
hours, in type B spermatogonia, a lit
shorter, about 13 hours, in intermedia
and gradually shorter from the 4th (1
hour) to the first (7-hour) type A gene
tion. The post-DNA synthetic time sho
the reverse pattern. It is very short, abo
4½ hours, in type B spermatogonia,
little longer, about 6 hours, in intermedia
spermatogonia, and gradually longer fro
the 4th (8-hour) to the first (14-hou
type A spermatogonia generation.

Alexander[3] : Do you feel that int
phase death of lymphocytes is similar
the type of interphase death you have stu
ied in these spermatogonia and oocyte
Secondly, are there any other cells th
you know of besides lymphocytes, thym
cytes, and the two germ cells you spo
about which show interphase death at lo
doses? Do you have any reasons that wou
lead you to think that the mechanisms

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[2] Valerio Monesi, Oak Ridge National Labo
tory, Oak Ridge, Tennessee.

[3] Peter Alexander, Chester Beatty Research
stitute, London.

rphase death of lymphocytes are simi- o the mechanisms of interphase death he spermatogonia and oocytes which have just been telling us about?

AKBERG: I don't know about the death ymphocytes in interphase. I imagine e of this is the same as what we ob- ed here. *In vivo,* in the intact animal, have a special problem in the lymph- e as we all know, in the stress syn- me. How this may be involved, I don't w.

LEXANDER: Does the stress syndrome rate on the germ cells you have studied?

AKBERG: To our knowledge, no. The evidence is that local radiation to the es alone is the same as total body ex- ure, precisely the same. There is an r in the literature on this point, but t has been corrected.

ow to get to your other question: Yes, is well known in other cells. The first k on this was done in Glücksmann's oratory, on tadpoles.

VANS[4]: This brings me to a point. ually where in fact do your cells die? icksmann says his cells died in late erphase. Can you get anywhere near s?

OAKBERG: If one has ever looked at se dead cells, when they get to the point t you can see they are dead and then when they started to degenerate, one n appreciate the difficulties of precisely termining this point. Let's put it some- ere in interphase, or in very early pro- ase.

EVANS: They made the point that they d two types of cell death. Firstly, death e to what they called the "ordeal of tosis," that is when cells got as far as tosis they collapsed and died. And sec- dly that most cells didn't quite make mitotic barrier, but died in late inter- ase.

OAKBERG: I agree. This brings up an- er point, namely, the study of mitotic lex in any cell system like this, where large number of cells die before they r divide. Some type A spermatogonia l appear perfectly normal for about 3½ ys, then degenerate just before they er their first post-irradiation division. us while most spermatogonia die and npletely disappear within 20 hours, a significant proportion of the surviving cells after this time may appear normal, but will degenerate before division occurs. Accordingly, data on mitotic index of irradiated spermatogonia must be interpreted with extreme caution.

KAPLAN[5]: Do you know whether they succeed in replicating a full complement of DNA before they die, or is there some indication that the DNA replication system in this particular instance is involved?

OAKBERG: Dr. Monesi will correct me if I am wrong. The cells that have completed DNA synthesis are more resistant to radiation than those that have not yet begun. So I think this brings the spermatogonia in line with other cells.

MONESI: The spermatogonia of the mouse seem to be much less sensitive to radiation following completion of DNA synthesis than during or before DNA synthesis. This is shown by injecting tritiated thymidine intraperitoneally into mice one hour prior to irradiation with X rays and scoring the number of labeled and unlabeled degenerated spermatogonia at various time intervals after radiation in autoradiographs. The different lengths of post-DNA synthesis times could account for the greater X-ray sensitivity of type B spermatogonia, in which the radioresistant post-DNA synthetic time is very short (4½ hours) and the radiosensitive DNA synthetic time very long (14½ hours). Variations in DNA synthetic time and post-DNA synthetic time also could be related to the heterogeneity of the X-ray sensitivity of type A spermatogonia, because the type A population is constituted of 4 cell generations each having different durations of the X-ray sensitive and of the X-ray resistant period of the cellular cycle.

VON BORSTEL[6]: Even at the more resistant stage, do the cells die before going into metaphase?

OAKBERG: Yes, they die the same way, but one can swamp this out with high doses. If you raise the dose high enough you kill all of them regardless.

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[5] Henry S. Kaplan, Stanford University, Stanford, California.

[6] R. C. von Borstel, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

KELLY[7]: To complete the answer to Dr. Alexander's question, there is one very interesting class of cells which seems to have the same type of interphase death. As Dr. Hicks and others have shown, that is a large group of embryonic neural cells which die within a few hours after radiation. This seems to be the source of the major abnormalities if embryos are irradiated. I think that eventually it will be proven that many bone marrow and intestinal cells also suffer interphase rather than reproductive death.

OAKBERG: Yes, there are other tissues, too, where this occurs. I think if this were looked for, it would be seen in a wide range of germinative tissues—not only the embryonic nerve cell.

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ley.

ect of Radiation Dose Rate on Mutation in Mice

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recent publication (Russell, Russell, Kelly, '60) summarizes much of our on the effect of radiation dose rate utation frequency. The present paper usses the results so far obtained in experiments that have not yet been pleted. Details of these experiments be reported elsewhere: it seemed more opriate, here, within the framework of und-table discussion, to summarize the lusions that can be drawn, and to lge in some speculation concerning nature and the role of "recovery" proc- s in the dose-rate effects that we have rved.

he new experiments confirm the real- of a dose-rate effect on mutation fre- ncy and provide additional evidence the view that the effect is an intra- ular one and not a consequence of cell ction. Two of these experiments have discussed briefly elsewhere (Russell Kelly, '60; Russell and Kelly, '61). y showed that the mutation frequen- at specific loci in spermatogonia of e irradiated at intensities of 0.001 r/ ute and 0.8 r/minute, respectively, in two experiments, were significantly er than the mutation frequency ob- ed earlier from 90 r/minute irradia- . The total doses used were 86 r and r in the first experiment and 600 r in second. It may be recalled that the inal demonstration of a dose-rate ef- in spermatogonia and oocytes was ed on a comparison of 90 r/minute 0.009 r/minute intensities (Russell Kelly, '58; Russell, Russell, and Kelly,). In a third experiment, the mutation uency in oocytes irradiated at 0.8 r/ ute for a total dose of 400 r was found be significantly below that observed en the same dose was delivered at 90 r/ ute. Thus, all three experiments con- the existence of a dose-rate effect.

The second experiment described above provides the best evidence, to date, that the dose-rate effect on mutation in spermatogonia cannot be attributed to cell selection. In this experiment with 0.8 r/minute irradiation, the length of the temporary sterile period following irradiation was no shorter than that observed at the higher dose rate of 90 r/minute. It appeared that the amounts of spermatogonial killing must be similar at the two dose rates. This was confirmed by actual measurement of spermatogonial survival at different dose rates, and at several doses within each dose rate, described in the accompanying paper by Oakberg and Clark. Thus, reducing the dose rate from 90 r/minute to 0.8 r/minute markedly lowers mutation frequency, but causes little or no change in the amount of killing in the cell population in which the mutations occur. The dose-rate effect on mutation in spermatogonia is, therefore, not dependent on cell killing. This greatly strengthens the view that the lower mutation frequency obtained at lower dose rates is a result of an intracellular "recovery," repair or prevention process.

Additional support for this view comes from the oocyte experiment in which the mutation frequency from 0.8 r/minute irradiation proved to be significantly below that from 90 r/minute irradiation. The effect of a total dose of 400 r on fertility is similar at these two intensities. In both cases, the females produce an average of between one and two litters before becoming sterile. Thus, as with the spermatogonia, the dose-rate effect on mutation cannot be explained in terms of cell killing.

The above oocyte experiment also seems to rule out another factor that could conceivably have played a role, namely, follicle stage. The total time required to give 400 r at 0.8 r/minute is only about 8 hours, which is much less than the variation in interval between irradiation and first mating that occurs within experiments. Thus, there can hardly have been any significant difference in the follicle stages involved in the 0.8 r/minute and 90 r/minute experiments. Again, it seems likely that the dose-rate effect on mutation can be explained only in terms of an intracellular repair or prevention process.

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[1] Operated by Union Carbide Corporation for U. S. Atomic Energy Commission.

Having concluded that the evidence points strongly toward a repair or prevention mechanism, it is of interest to examine the results of the above experiments, and others, to see what additional information can be deduced about the nature of this presumed mechanism.

In the experiment (Russell and Kelly, '60) in which spermatogonia were irradiated at a dose rate of 0.001 r/minute, the mutation frequency is significantly higher than in the controls ($P = 0.002$). Thus there is no indication of a threshold dose rate, even at what is, as far as we know, the lowest dose rate ever tried in laboratory mutation rate experiments with animals. An additional point of interest is that the mutation frequency at 0.001 r/minute is not significantly different from that obtained at 0.009 r/minute. Thus, there is, so far, no evidence that lowering dose rate below 0.001 r/minute would result in any further reduction in mutation frequency. These data indicate that the repair or prevention process cannot eliminate *all* mutations in spermatogonia: approximately 25% of the mutations occurring at high dose rates cannot be prevented even when the dose rate is dropped to a very low level.

After many years of belief in the absence of a dose-rate effect on mutation, and in the existence of dose-rate effects on somatic damage, it is something of a paradox to find, in the spermatogonia experiment with 0.8 r/minute irradiation, that, as compared with 90 r/minute experiments, there is a large dose-rate effect on mutation and little or none on cell killing in the very same population of cells. At much lower dose rates, Oakberg and Clark ('61) have found clear evidence of
a marked dose-rate effect on cell killi
However, the conclusion would seem ju
fied that the processes that operate in
repair or prevention of the mutatio
steps are not working in parallel with
ones that promote recovery from soma
damage.

A similar lack of parallelism is fou
in the oocyte experiments. Data from
90 r/minute and 0.8 r/minute irradiati
(400 r total dose) show little differe
in their effects on fertility (oocyte killin
but a marked difference in mutation f
quency. At lower dose rates, fertility d
show a dose-rate dependence (Russe
Stelzner, and Russell, '59), and this agr
with effects on oocyte survival (Oakb
and Clark, '61).

As the data accumulate, there seems
be increasing evidence for a difference
the dose-rate response in spermatogo
and oocytes. Earlier results (Russell, R
sell, and Kelly, '60) already indicated t
the effect might be greater in oocytes. T
present trend in the results from 0.8
minute irradiation of oocytes suggests t
the mutation frequency may turn out
be intermediate between those from 90
minute and 0.009 r/minute irradiatio
This is in contrast to the present d
from spermatogonia, where 0.8 r/minu
and 0.009 r/minute results do not diff
significantly. Of course the spectrum
mutations in oocytes probably differs fr
that in spermatogonia: e.g., *d se* defici
cies seem to be common in the former a
not in the latter (Russell and Russe
'60). This must certainly be kept in mi
in making comparisons. More data a
needed before firm conclusions can
drawn, but the information already o
tained increases the probability that mal
and females will show different dose-ra
responses.

Since mutant genes are, in gener
quite stable, it seems reasonable to spec
late that, if the lowered mutation fr
quency at low dose rates is a result
repair, then this is probably not repair
a completed mutation, but repair of a p
mutational damage. Regardless of wheth
one visualizes the dose-rate effect as bei
due to repair of premutational damag
or to prevention of completion of the m
tational steps, the induction of mutati

nization is pictured as a somewhat ect process. This view is supported vidence from other organisms (see Kimball and Wolff, this volume). On basis one would expect to discover a greater number of factors affecting tion than was anticipated on the , simpler view in which dose-rate endence was a kingpin. Thus we ld not be surprised to find that cell is an important factor, not only in ting the qualitative nature of the mutions induced, but also in influencing things as the dose-rate response. hermore, some of the long established ngs need to be looked at from the point of view. The absence of a rate effect in spermatozoa may be as an example. This used to be reed as a basic phenomenon from which as safe to generalize to all germ cells. aps it should now be looked upon as odd phenomenon, restricted to sperozoa, and occasioned by the peculiar re of the specialized spermatozoon "Recovery" is a far more basic feature iological material, and the evidence ussed here indicates that it plays an ortant role even in the radiation inion processes of mutation in germ .

OPEN DISCUSSION

VANS[2]: I take it that these mutations lve some of the 7 specific loci which group and the Harwell group have studying. Is that correct?

USSELL: Yes.

VANS: Do you have any double mus? In essence, what are your mutas? Are they in fact point mutations or ll rearrangements such as deficiencies, lications, and so on?

he point behind the question is that, ey are structural rearrangements, then il to see how recovery can operate. nderstood the Harwell data include e double mutants which looked to me e suspicious in the sense that they ht be deficiencies.

USSELL: From the irradiation of postmatogonial stages and oocytes, we e frequently obtained double mutants he closely linked *d* and *se* loci. How-, from irradiation of spermatogonia, we have observed only one case of such a double mutant, in spite of a large number (over 60, as I remember it) of single *d* and *se* mutations. Thus, there is no evidence for deficiencies being common in one of the very cell stages in which we find a marked dose-rate effect.

In addition to this, in Drosophila, Alexander and Ward who, like us, worked on specific locus mutations induced in spermatogonia, looked cytologically at these mutations and found not a single deletion or other chromosomal aberration, whereas, with the same procedure in spermatozoa, a high proportion of the specific locus mutations were found to be associated with chromosomal aberrations.

VOICE: In line with Dr. Evans' question, are any of your mutations lethal when homozygous?

RUSSELL: About 75% of the specific locus mutations in spermatogonia are homozygous lethal.

EVANS: It looks suspicious, doesn't it?

RUSSELL: Not necessarily. Alexander and Ward also found a high proportion of lethals, and these, as I have just mentioned were not associated with chromosomal aberrations. I think Dr. Valencia could contribute something interesting on this point.

VALENCIA[3]: We have analyzed 58 mutations at 10 specific loci on the X chromosome of Drosophila, induced by X irradiation of females. Of 15 mutations induced in mature oocytes, 12 were associated with chromosome aberrations, mostly small deficiencies. On the other hand, of 17 mutations induced in oogonia, although 10 were lethals, only three were associated with detectable chromosome aberrations (two deficiencies and one translocation). Now it is possible that these three aberrations were recovered from cells which actually were beyond the gonial stage at the time of treatment, but which were somehow held in the ovariole longer than usual and were tested with the gonia. At any rate our data agree that aberrations are either never or at most very rarely recovered from gonia.

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[2] H. J. Evans, Brookhaven National Laboratory.

[3] R. M. Valencia, Argentine Atomic Energy Commission.

LÜNING[4]: May I ask Dr. Russell whether the lethals are occurring mostly shortly after the sterile period, or if the lethals are distributed equally from mutations obtained from the first and the second half of that period?

RUSSELL: I suspect that they are about equally distributed. I haven't made a tabulation on this point, but from looking at the data as they come in, I am pretty sure the lethals are about evenly distributed.

AUERBACH[5]: I don't understand Dr. Evans' question. I think it raises the whole problem of what is recovery. There is no reason why the prevention of the termination of some event should not occur equally well with chromosome breakage or reunion as with mutation. Furthermore, even if there are deficiencies, they are not necessarily true rearrangements.

EVANS: What I did not understand was the fact that Dr. Russell, when he talked, was generally siding towards the idea that he had some effect which did not fit in with what Dr. Wolff was giving us this afternoon in the way of chromosome breakage, but rather was more in accord with the ideas that Witkin and Doudney were presenting on mutations in bacteria. That is a recovery system similar to that operating in UV treated bacteria.

OSTER[6]: A major significance of Dr. Russell's work lies in the fact that he has found such a recovery process taking place for point mutations, because it had already been known that fractionation of the dose does allow time for breaks to recombine before others are produced. Therefore, by fractionating the dose you get few chromosomal disarrangements.

EVANS: Yes, but that is a recovery we know about. We can understand it.

RUSSELL: What I should like to emphasize again is that we find a dose-rate effect in a cell stage in which, according to our evidence, the specific locus mutations scored are only quite rarely associated with chromosomal aberrations, including quite small deficiencies. Thus, as Dr. Oster has just mentioned, we think we have evidence for a dose-rate effect different from the well-known one which can be accounted for in terms of multiple chromosome breaks.

FAILLA[7]: I would like to call atten
to an old experiment that was carried
by Dr. Henshaw some 30 years ag
which he found recovery in the cas
Arbacia eggs and no recovery in the
of the mature sperm. This is not a ge
experiment. I will tell you in a few w
just what the experiment was about.

At room temperature the *Arbacia* f
lized egg divides in about 50 to 60 min
If you irradiate the eggs alone, say
10,000 or 15,000 r, and then fertilize t
with non-irradiated sperm, instead of
first division occurring in 50 minute
may take 100 minutes.

Now, if instead of fertilizing imm
ately after completion of the irradia
you wait, say, half an hour, then
cleavage delay is very much less t
when the eggs are fertilized immediat
This was called recovery. The wor
described in Lea's book and I think m
of you have read about it. If you do
same thing with the sperm, you can
the delay in the time of first cleavage
it is almost the same as when the e
are irradiated with the same dose.
there is no recovery. In other words
you irradiate the sperm and then v
1 hour before inseminating non-irr
ated eggs with it, you get the same amo
of delay that you would if you insemin
the eggs immediately after irradiating
sperm.

So this is, let us say, a demonstrat
of recovery within the cell itself in
case of the egg and no recovery in the c
of the sperm. The difference perhaps
that one has a lot of cytoplasm and
other one doesn't and the metabolism
quite different in the two.

If there is any similarity with the ef
that Dr. Russell is discussing, the dif
ences may also be attributable to the ab
dance of cytoplasm in the case of sp
matogonia, and to the near absence
cytoplasm in the case of mature sp
matozoa.

AUERBACH: I think we will have to
over to *Drosophila* now. I first want

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[4] K. G. Lüning, University of Stockholm.

[5] Charlotte Auerbach, Institute of Animal netics, Edinburgh.

[6] I. I. Oster, The Institute for Cancer Resea

[7] G. Failla, Argonne National Laboratory.

y to discuss the evidence for similar-
etween these results and *Drosophila*
s.

onder whether Dr. Meyer in no more
5 minutes could say what she did
r fractionation experiments?

YER[8]: We were using this method
urely practical reasons only, to see
er we could give a very high dose of
s and not lose most of the germ cells
e "killing effect," since fractionation
es the incidence of chromosomal
ations and allows much regeneration
the intervals between treatments are
enough. We gave repeated treatments
00 r each, with 4-day intervals. As
ted, we found that such fractionation
ot alter the additive dose-mutation
relationship. We obtained 5.3% of
nked lethals after 12,000 r, an inter-
ate value of 8.6% after 16,000 r, and
% for 24,000 r. This is also in agree-
with some later results, and corre-
ds to a mutational response of ap-
mately ½% of sex-linked lethals per
r.

ERBACH: How long was the interval?

EYER: These experiments used inter-
of 4 days. One can shorten this inter-
omewhat.

ICE: What did you irradiate?

EYER: We irradiated adult flies.

ICE: Male or female?

EYER: Females, testing germ cells
h had been in gonial stage during
entire irradiation period. Later on,
ame was done with males, using total
mulated doses ranging from 8000
6,000 r, and looking for autosomal
ls.

ERBACH: I want to add that, at Har-
there has been a test of the effect of
rate on the production of autosomal
ls in *Drosophila* males. I have seen
anuscript before it went to press, but
't have it here to check on the details.
I know that in some experiments there
no effect at all of dose rate. In one
o there was a very slight effect. (Note
d in proof: New results from a more
al experiment show a marked effect
se rate. These appeared in a revision
he manuscript.) The paper should
e out in the International Journal of
ation Biology. Incidentally, in this work there was a definite difference between cobalt gamma and X rays in mutation frequency.

It is so late now that I can only say a few words about Dr. Sobel's results with various post-treatments after irradiation. Here he used nitrogen, potassium cyanide, and chloramphenicol. The results with potassium cyanide are contradictory. Post-treatment with nitrogen gave the same result that Dr. Abrahamson found; it enhanced the effect of the irradiation. Possibly this is restricted to breakage events, breaks remaining open for a longer time. With chloramphenicol treatment, he got a decreased mutation frequency in early spermatogonia.

I think we should now go on to the last two points which belong together. These are the differences in mutation frequency between mature or almost fully mature spermatozoa in *Drosophila*, depending on the day after irradiation on which they are utilized or on the environment in which they are irradiated. These results can be variously interpreted as recovery or as differences in sensitivity.

Dr. Lüning has new data on the first point.

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[8] Helen Unger Meyer, Indiana University.

covery from Premutational Damage of X Irradiation Drosophila Spermatogenesis[1]

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has been observed that posttreatment cyanide results in a significant in- se of the frequency of sex-linked ls in *Drosophila melanogaster* if ex- d to X rays at a high-dose rate of 2200 nute (Sobels, '57, '58). The effect is icted to stages with peak sensitivity e mutagenic action of radiation, pre- ably corresponding to spermatids or matocytes. Evidence was obtained that ncreased lethal frequency due to cyanide posttreatment refers to gene muta- s and possibly small deletions.

explain these observations, I had nally considered the possibility that accumulation of radiation-produced xides might be responsible for the treatment effect. This hypothesis is supported, however, by the finding at doses producing the same genetic t, posttreatment is equally effective ising the mutation rate, whether high- nsity radiation is given in either pure gen or oxygen. As an alternative in- retation I have assumed that cyanide rferes with a recovery mechanism ch in the absence of cyanide restores of the premutational radiation dam- (Sobels, '60).

he present communication concerns a her analysis of the cyanide effect. In ection with the recovery hypothesis lso studied the effect of posttreatment nitrogen, that of dose fractionation , or without, nitrogen posttreatment, the action of chloramphenicol on the uencies of radiation-produced muta- s in different stages of spermato- sis.

METHODS

nce lethals represent a heterogeneous gory of genetic effects, a ring-shaped romosome was uniformly used for these experiments. This procedure greatly increases the resolving power for detecting lethals originating as point mutations, or at least as one-hit effects. The mutational response in successive stages of spermatogenesis was studied by remating the treated 1-day old X^{c2}, yB males every 2 days to 3 and in later experiments to 6 fresh females of the genetic constitution $y\ sc^{S1} In\ 49\ sc^{8}$.

In almost all experiments, X irradiation was given by means of an ENRAF-machine at 100 kv, 4 mA at a dose rate of 45 r/second with 1 mm A1-filtration, or at an intensity of 55 r/second without additional filtration. For the chloramphenicol experiments a lower dose rate of 565 r/minute was used.

Hydrocyanic acid was applied at a constant rate of flow of 100 ml/minute during 8 minutes at a concentration equivalent to 35 mg KCN. The nitrogen was purified by passing it through washing flasks with sintered bottoms, containing "O_2-multirapid" (Unionapparate Baugesellschaft M.B.H., Karlsruhe). For details of chloramphenicol concentrations see section IV.

RESULTS

I. *Posttreatment with cyanide*

For a further analysis of the effect of cyanide, we aimed at a more regular and pronounced expression of its modifying action on radiation-induced mutational damage. Cyanide was given repeatedly after fractionation of a total dose of 1525 r into 5 fractions, separated by 2-hour intervals between the fractions; each fraction followed by cyanide-posttreatment.

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[1] These studies were carried out with support from the Health Research Council T.N.O. and the Institute for Radiopathology and Radiation Protection.

This procedure, however, did not change the cyanide effect. In comparison to the mutagenic effect of unfractionated radiation only, posttreatment in both the fractionated and the unfractionated group of flies resulted in a significant decrease of the mutation rate in stages with peak sensitivity. For the presentation in figure 1, left, data of the two posttreated groups are pooled. In contrast to earlier experiments, both radiation and cyanide were given to unetherized flies, and the interval between the two agents was considerably shorter.

A separate analysis in a subsequent experiment showed, however, that neither etherization, nor changing the interval between radiation and posttreatment from 2 seconds to 2 minutes changed the cyanide effect. However, in this particular experiment (see fig. 1, right) flies that received radiation only, showed an abnormally low mutation rate in stages with peak sensitivity, resulting in a highly significant enhancement in the posttreated groups. It is possible that an irregular release of germ cells in the successive broods, resulting in an admixture of stages with widely different sensitivities was responsible for this kind of undesirable variation. For a subsequent experiment, in which the effect of posttreatment with cyanide was studied at three different doses of about 800, 1600, and 2000 r respectively, 6 females per male per brood w
therefore used.

The results of these experiments
shown in figure 2. They can be summ
ized in the following manner:

1. *Effect of dose.* We observed t
after exposure to radiation only, the r
tation rates in mature sperm (brood
and spermatogonia (brood e) incre
linearly with dose.

2. *Separation of stages.* We noted t
after exposure to 1600 r, sterility sets in
brood c and is almost complete in brood
After radiation with 2000 r, both bro
c and d are sterile, whereas at the lo
dose of 800 r fertility in brood d is o
slightly lowered.

3. *The most sensitive stages.* We s
that after radiation with 800 and 160
the highest mutation rates occur in
most sterile broods, suggesting that sp
matocytes and possibly early spermat
were sampled. The observation that af
exposure to 2000 r the mutation rate
these stages is lower than after a dose
1600 r, suggests that, at the high do
cells with maximum sensitivity to
mutagenic action of radiation have be
selectively eliminated.

4. *Effect of posttreatment.* It is cl
that after exposure to 800 r, cyanide lo
ered the mutation rate in brood d; th
after radiation with 1600 r a pronounc

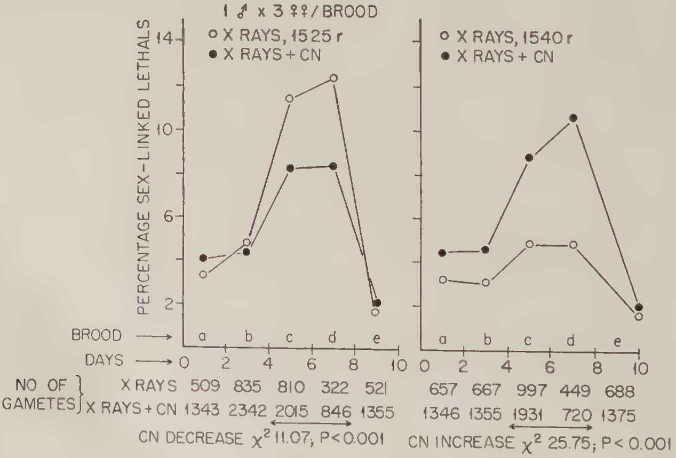


Fig. 1 Posttreatment with HCN following exposure to high-intensity X irradiation may result in either a decrease (left) or an increase (right) of radiation-induced mutation frequencies in stages with peak sensitivity; for further details see text.

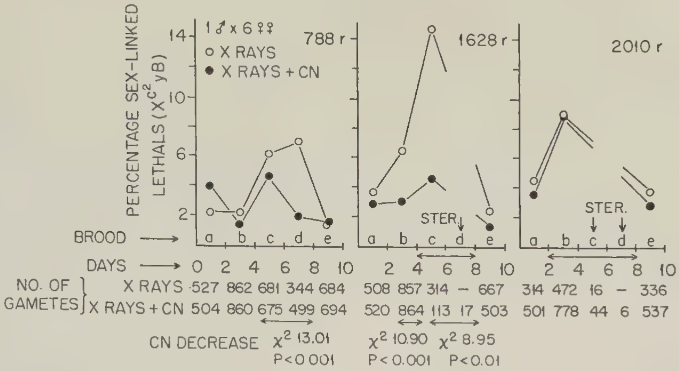


Fig. 2 The effect of posttreatment with HCN after exposure to three different doses of
igh-intensity X irradiation, using an efficient mating procedure for separation of the suc-
essive stages of spermatogenesis.

of the mutation rate occurs in both
ds b and c; and that at the high dose
00 r the cyanide effect does not show.
posttreatment remained without ef-
at the high dose, presumably results
a selective elimination of stages where
ide can specifically modify the initial
tion damage.

he observation that with this appar-
efficient separation of stages with
rent radiosensitivity, posttreatment re-
d in a lowering of the mutation rate
have different interpretations. If, on
basis of earlier data, one assumes that
also cyanide inhibits a recovery proc-
then the lowering of the mutation
after posttreatment, as observed in
more recent experiments, requires fur-
interpretation. It is possible that a
e efficient sampling of stages where
ide operates has resulted in a propor-
ally greater selective elimination of
with mutations. It was thought that
is interpretation was correct, the se-
ve effect would not show after expo-
to a low dose of radiation and that
igher doses, exceeding 800 r, post-
tment would result in an increase of
frequency of dominant lethals. How-
, after exposure to a low dose of 400 r,
treatment did not result in an increase
he frequency of sex-linked recessive
als and dominant lethality, following
ation with a dose of 1620 r was like-
not affected by posttreatment. These
lts then clearly do not support the
that selective elimination of cyanide-sensitive cells resulted in a spurious lowering of the frequency of sex-linked lethals in the experiments shown in figure 7-2.

It would seem that the observations after posttreatment with cyanide can be plausibly explained, if, in analogy to the observations of Kimball, Gaither and Perdue ('61) in *Paramecium*, one assumes that cyanide may affect two contrasting processes, one being associated with the rate of disappearance of premutational damage, the other with the time, or rate required for its fixation. Inhibition of the first process, that is inhibition of metabolic repair will result in an increase in the amount of mutation. Alternatively, prolongation of the time interval needed for fixation, or slowing down the rate of the fixation process, will result in a decrease of the mutation-frequency. If, depending on hitherto unknown experimental conditions, the effect of cyanide is more pronounced on either one, or the other process, both an increase and a decrease of radiation-induced mutation rates may be observed.

II. Posttreatment with purified nitrogen

In view of the observations after posttreatment with cyanide, we were interested to see whether other posttreatments interfering with oxydative respiration would be likewise effective in affecting radiation-induced mutation rates. Immediately following radiation with 1450 r, given at a dose-rate of 55 r/second, flies were exposed to a flow of pure nitrogen for 25 minutes. Data from two experiments showed that posttreatment with nitrogen results in a significant increase of the radiation-induced mutation frequencies in stages with peak sensitivity and in earlier stages. The pooled data are presented in figure 3. It would seem, therefore, that anoxia following high-intensity irradiation interferes with recovery processes in a way similar to posttreatment with cyanide.

III. *The effect of dose fractionation*

A number of fractionation experiments were carried out to investigate whether dose fractionation would favor recovery after application of high-intensity radiation. The mutagenic effects of a total dose of 1350 r that was given either at once, or in 5 fractions with intervals of 2 hours between the separate fractions, were studied in 7 successive 2- to 3-day broods. In order to avoid faulty dosimetry, the flies were confined in a very small space, so that an ionization chamber could be mounted immediately on top of the radiation vessel. To prevent anoxia, a stream of air was passed over the flies before and during the irradiation.

In total, three comparable experiments have been carried out. In two of these the mutation frequencies in stages with

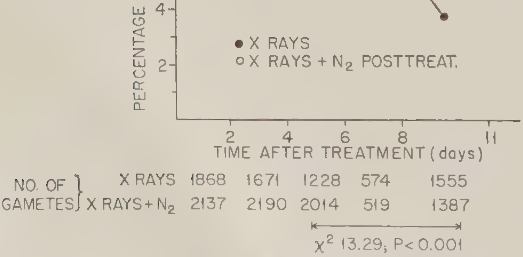


Fig. 3 The enhancing effect of posttreatment with purified nitrogen on the frequencies of mutation induced by high-intensity X irradiation in stages with peak sensitivity and earlier stages.

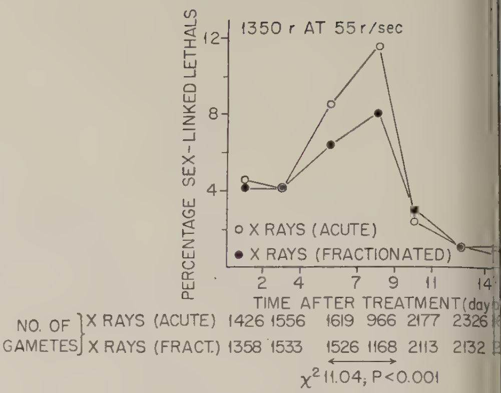


Fig. 4 The lowering effect of dose-fracti
tion on the induction of mutations by h
intensity X radiation in stages with peak se
tivity.

peak sensitivity, as sampled in brood
and d, were significantly lower in the fr
tionated than in the acute group.
pooled data of these experiments are
sented in figure 4. The data also sh
that fractionation did not affect the ind
tion of mutations by irradiation in eit
mature sperm (broods a and b) or sp
matogonia (broods e, f and g). In a th
experiment, fractionation was without
fect for no apparent reason. Since,
this particular experiment 4-hour
males were used, instead of 24-hour ma
as in the other positive experiments, i
possible that this difference influenced
fractionation effect.

In a fourth experiment, we stud
whether posttreatment with nitrog
would modify the fractionation eff
The results showed that with nitrog
posttreatment, dose fractionation likew
results in a significant decrease of
mutation frequency in broods c and
where peak sensitivity was encounte
(fig. 5). Moreover, as in the experime
on nitrogen posttreatment, the effect
tended to an earlier stage of germ-cell
velopment than that exhibiting peak s
sitivity.

In view of the fact that after fractio
tion of the dose in three independent
periments a significant reduction of
mutation frequencies was encountered
stages with peak sensitivity, we are
clined to believe that this is a real eff

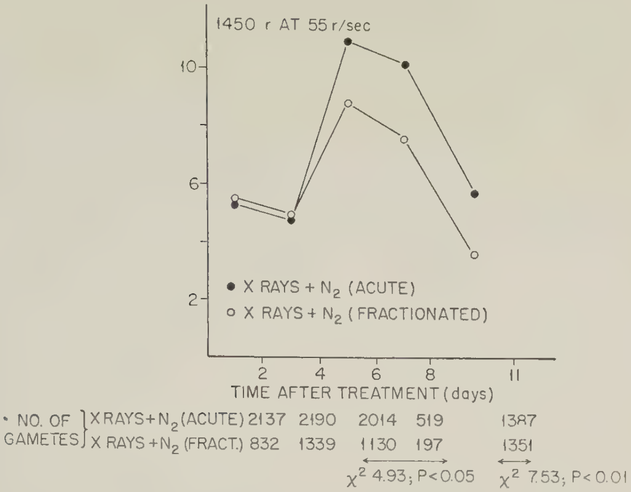


Fig. 5 The effect of posttreatment with purified nitrogen, following high-intensity radiation given in 5 fractions, separated by 2-hour intervals, between the fractions, as compared to that of nitrogen following unfractionated exposure.

eems of particular interest that frac-
ation of the dose reduces the mutation
uency exactly in those stages where
enhancement was observed in the ear-
experiments on posttreatment with
nide. The results of the fractionation
eriments therefore support the hypoth-
that in these stages physiological proc-
es are operating that are responsible
partial recovery of the initial radiation
nage.

n connection with the intensity effect
spermatogonia of the mouse (Russell,
ssell, and Kelly, '58, '60) and *Drosophila*
ster, Zimmering and Muller, '59), it is
prising that a fractionation effect was
observed in gonia. It is admitted that
-linked lethals in a ring-chromosome
unsuitable material for a proper analy-
of bunches of identical mutations. The
umption, however, that the bunches ob-
ved in these early stages were due to
mutation only, did not change the
ults. It is possible that the discrepancy
ween our fractionation data and those
intensity effects of other authors is due
the very high intensity of radiation in
present experiments. This variable
y have influenced the repair processes
an unfavorable manner (cf. Russell,
ssell, and Kelly, '58).

IV. *The effect of pretreatment with chloramphenicol*

The action of chloramphenicol on the mutagenic effects of radiation in different stages of spermatogenesis was studied with two purposes in mind. First, studies of Kimball, Gaither and Wilson ('59, a, b), in *Paramecium* and of various authors in bacteria (Witkin, '56, '58; Doudney and Haas, '58; Alper and Gillies, '60; Lieb, '60) have shown that inhibition of protein synthesis plays a part in recovery from radiation damage. Second, it has been proposed that changes in the composition of proteins during the transition from spermatids to mature sperm (Felix, Fischer, and Krekels, '56; Alfert, '59) might be responsible for the differential radiosensitivities of these two stages (Oster, '59). Since posttreatment with cyanide, as well as dose-fractionation are only effective in modifying the mutational radiation damage in spermatids and not in sperm, a clarification of the nature of the differential radiosensitivity of sperm and spermatids seemed of particular interest.

Three sets of experiments were carried out in which the flies were injected with concentrations of chloramphenicol, ranging from 0.258–0.0108 mg/gm live weight, preceding exposures from 1200–1800 r. Since the data of the separate experiments did not differ significantly with respect to the effect of chloramphenicol, the data were pooled; they are shown in figure 6. Considering the results in the separate broods it can be noted that:

1. In brood a, representing the most advanced stage of mature sperm, chloramphenicol causes a slight, but significant increase of the mutation frequency.
2. In brood b chloramphenicol has no effect.
3. In stages with maximum sensitivity, as sampled in broods c and d, corresponding to spermatids and meiotic stages, pretreatment with chloramphenicol results in a highly significant decrease of the mutation rate.
4. In stages, sampled in brood e, probably corresponding to late spermatogonia, chloramphenicol likewise reduces the radiation-induced mutation frequency in a significant manner.
5. In earlier spermatogonia (brood f) chloramphenicol has no effect.

The results obtained in mature sperm and spermatids suggest that indeed protein synthesis plays a part in the origin of the different radiosensitivities of these two stages. This idea is further supported by similar observations after pretreatment with ribonuclease (Sobels, '61).

It is of interest that pretreatment with chloramphenicol is effective in reducing the radiation-induced mutation freque cies in spermatids, since for this sta indications for repair processes were tained both after posttreatment with anide and nitrogen and in the dose-fra tionation experiments.

The observation that chloramphenic has an effect in meiotic cells (brood and in spermatogonia (brood e) is not surprising, since in these stages cell di sion goes on and consequently prote synthesis may be expected.

SUMMARY

The data, relating to repair processes *Drosophila* males are summarized figure 7. The subdivision in stages at th bottom is tentative and serves only to he orientation. Weighted mean frequenci of mutations in the successive stages aft exposure to radiation alone are shown the top. They were taken separately standard for each group of experimen and their relative modifications by fra tionation or chemical agents are show as horizontal bars extending over one more stages.

Taken together the *Drosophila* data pr sented here suggest that repair from pr mutational damage is possible in at lea three different stages of spermatogenesi that is spermatids, meiotic stages and la spermatogonia. Most data are availabl

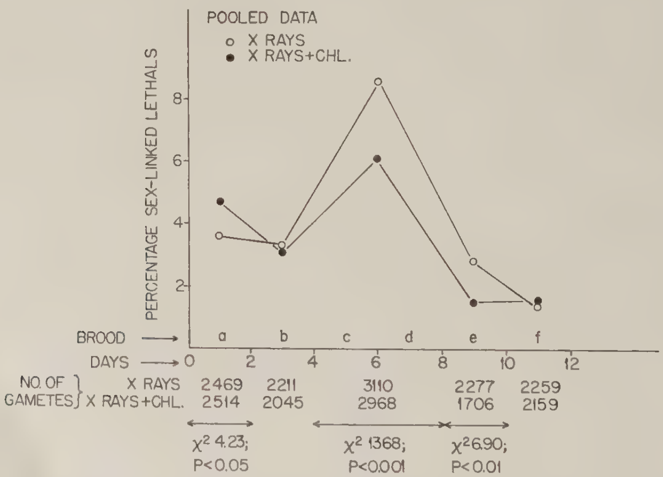
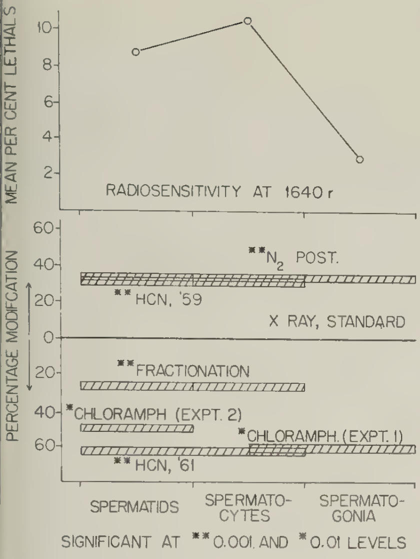


Fig. 6 The effect of pretreatment with chloramphenicol on the frequency of mutation induced by X irradiation in successive stages of spermatogenesis. Pooled data from 3 sets of experiments using 2-day brood changes.



g. 7 A summary of the data on repair of
ational radiation damage in *Drosophila* sper-
ogenesis. Subdivision in stages at the bottom
hematic. The lower diagram shows the per-
ual modification of the mutation rates, after
fractionation and treatment with the dif-
nt chemicals, in comparison to the mutagenic
n of radiation alone. The top graph shows
hed mean lethal frequencies after exposure
640 r as a measure for differential radio-
itivity in the stages under consideration. The
for chloramphenicol only refer to those ob-
ed in the first two experiments (chl 1 and
2). A more complete picture of the effects of
ramphenicol is shown in figure 6.

stages characterized by peak sensitivity
K irradiation, presumably consisting of
rmatids and late spermatocytes. Here
ctionation of the dose and pretreatment
h chloramphenicol lower the mutagenic
ects of radiation, whereas posttreatment
h nitrogen results in an increase of
iation-mutagenicity. Posttreatment with
nide may result in either an increase
a decrease of the mutation-frequency.
pointed out before, these observations
be explained by assuming that two
cesses with contrasting effects are in-
ved in repair from premutational dam-
. The observation that pretreatment
h chloramphenicol results in an in-
ase of the radiosensitivity in sperm and
a decrease of that in spermatids may
p to understand the pronounced dif-
ences in radiosensitivity between these
stages (Sobels, '61).

Secondly, recovery seems to occur in dividing cells as meiotic stages and late spermatogonia where the mutagenic effect of radiation is reduced by pretreatment with chloramphenicol and enhanced by anoxia, following radiation. The observation that in dividing germ cells of *Drosophila* completion of the radiation-initiated mutation process is associated with protein synthesis is in good agreement with the findings in lower organisms like *Paramecium* and bacteria. Also, it may be pointed out that there is a fair correspondence between the course of spermatogenesis in *Drosophila* and in the mouse. In the mouse the work of Russell and coworkers gives a clear indication that repair of initial radiation damage in gonia is possible. In view of the present *Drosophila* data it is tempting to believe that there is a uniform pattern to which repair-processes conform in dividing cells of widely divergent organisms.

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n *Drosophila* Spermatozoa be Used in Studies Recovery Processes?

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he word “recovery” has been frequently l in radiobiology in recent years. Vari- authors have meant different things his common word with a consequent usion. For that reason it is necessary omment on the present use of the word. ı this paper, I am following my own and use the word recovery as I had l it earlier in some papers. To me the d recovery will mean those processes ch interact with an initiated mutation cess in such a way that it switches back ormal or near normal conditions. This nition will cover both influence upon omosome breaks and upon point muta- s.

ly interest in the recovery process de- d from a series of experiments with *sophila* males irradiated in air or in ogen (Lüning *et al.*, ’57, ’58, 59). When dose was divided into two fractions, could be given in nitrogen, the other air. If the fraction given in air was re than 1100 r and the interval between two fractions was not more than 30 nutes the effect was the same as if the ole of the dose had been given in air. these results were obtained from males ted within the first 24 hours after irradion. This is a very important fact as will seen below.

From my results I concluded that irradion of sperm—to be used within 24 rs after treatment—induced the same e of primary damages to the chromones in nitrogen and in air, however, en the entire dose was given in nitrogen ne of the primary damages were preited from reaching the ultimate state ectable as a mutation in its widest aning. A similar effect was also obved in the case of fractionation if the erval was more than 30 minutes. In these cases the effect was intermediate and equal to the sum of the two fractions.

This effect after irradiation in nitrogen in my interpretation will mean a recovery process wherever the primary damages are prevented from ending up as point mutations or chromosome breaks.

Wolff and Lindsley (’60) confirmed my results as to comparison between irradiation in air or nitrogen but found a higher rate of breaks after irradiation in pure oxygen. Furthermore, they learned that fractionation with doses in nitrogen and oxygen was less effective than the whole of the dose in oxygen. According to Oster (’57, ’58) spermatozoa are relatively anoxic compared to other stages. This statement is, however, not unequivocal. Baker and von Halle (’53) showed that there is a difference between sperm inseminated the first 24 hours after irradiation compared to the second day if the irradiation was given in air but that no differences were observed when the irradiation was in nitrogen atmosphere. These observations have been substantially confirmed in my own work. As it is of importance to know the relationship between these two groups of sperm (first opposed to second day) I will present a material in which 3–4 day old $M5;\ sc^{8}Y$ males ($M5 = \text{Muller-5} = sc^{S1}\ InS\ w^{a}\ sc^{8}$) were irradiated in air or in nitrogen atmosphere. Within 2 minutes after the treatment the males were mated for 24 hours to *y w sn* females and were then transferred to a new group of virgin females for another 24 hours mating. After mating the females were allowed to oviposit for two 3-day periods. The results are presented in table 1. From series 1 and 2 it is obvious that a marked difference between the two mating periods exists for both types of muta-

TABLE 1

Mutation differences between first- and second-day sperm

Series	Treatment	Mating period	Total F_1	XO ♂	% XO ♂	F_1 ♀	Yellow	10^{-4} Yellow
		hours						
1	3300 air	0–24	96345	865	0.90	49529	143	28.9
2	3300 air	24–48	105550	733	0.69	55173	89	16.1
3	3300 N_2	0–24	116214	697	0.60	60982	79	13.0
4	3300 N_2	24–48	102438	617	0.60	53361	69	12.9

tions although in series 3 and 4 there is no difference. This means that in the second day sperm there was a much slighter effect of the change in atmosphere than in the first day sperm. The second day sperm might be rather anoxic but the first day sperm are much less anoxic.

The courtesy of one of my colleagues, Dr. Ruth Sävhagen, made it possible for me to show a comparison between the effect of irradiation of 3–4 day old *Drosophila* males in pure oxygen as opposed to air. Her main interest was the period 5–10 days after irradiation but the data described also a mating during the first 4 hours postirradiation. From the 4th day the males were mated daily to new groups of females. Table 2 presents the ratios between the oxygen and the air series. More than 260,000 flies were used in each of these series. It is obvious that the effect of oxygen was at least not more drastic in the sperm used the first 4 hours than in the other stages, including spermatids and spermatocytes.

The reason for using a mating period as short as 4 hours needs some further comments. The original finding of Baker and von Halle ('53), that there was a di ference between the first and the secon day sperm, did not tell anything abou when the low-yielding sperm could b available. A partitioning into 24-hou periods is a conventional one. It was consequently of great interest to get the pa tern of the change from high to low yielding sperm. This has been analyzed fo males irradiated both when 0–1 day ol and 3–4 days old. The males were mass mated in plastic boxes for short periods i which the mating time was delimited with in ±1 minute. In some cases the male were mated immediately after treatment in others they were stored for some hours After the mating the females were allowe to oviposit for two 3-day periods. The results are presented in tables 3 and 4.

From table 3 it is evident that mating started a few hours after irradiation had higher yield of aberrant males than i matings started later on. As early as 3– hours after irradiation the low yielding sperm was available. In table 4, representing males irradiated when 3–4 days old the low yielding period did not come unti 12 hours after irradiation. Without presentation of further data, it suffices to sa that it was irrelevant whether the 3–4 da old males had been stored with or withou females from eclosion to irradiation.

Summing up, these results indicat heterogeneity in sperm available the firs day after irradiation and, that the tim when the two groups of sperm are avai able depends upon the age of the males

If one wants to study the recovery process it is necessary to be aware of thi heterogeneity otherwise the effects migh be obscured. From my earlier discussion possible conclusion is that the differenc between sperm available the first few hou after irradiation compared to those fro

TABLE 2

Mating period	Ratio[1]
0–4 hr.	1.19
4th day	1.60
5th day	1.21
6th day	1.27
7th day	1.39
8th day	1.55
9th day	1.89
10th day	1.55

[1] Ratio: $\dfrac{\text{Rate of XO males induced in } O_2\text{-atmosphere}}{\text{Rate of XO males induced in air atmosphere}}$.

The males were irradiated when 3–4 days old. Dose 1100r. Data from Sävhagen ('61).

TABLE 3

–1 day old cv:sc^8 Y males were given 3300 r X rays and were then mated to y w sn females for 1 to 3 hours either immediately or after a varying number of hours. XO males and total number of offspring as well as the rate of XO males are given.

Series	Mating period after irradiation	XO males	Total offspring	% XO males
	hours			
1	0–1	3	446	0.67
2	0–2	58	5655	1.02
3	0–3	140	13617	1.03
4	1–3	47	3374	1.10
5	1–4	72	7346	0.98
6	2–4	31	3742	0.83
7	3–5	44	4331	1.02
8	3–6	82	11047	0.74
9	6–9	85	10802	0.79
10	9–12	165	21738	0.76

TABLE 4

3–4 day old cv:sc^8 Y males kept without females for 3 days prior to irradiation. Otherwise see table 3.

Series	Mating period after irradiation	XO males	Total offspring	% XO males
	hours			
11	0–1	94	10674	0.88
12	0–2	325	31765	1.02
13	0–3	407	38163	1.07
14	1–4	173	17290	1.00
15	2–4	254	25331	1.00
16	3–6	90	9561	0.94
17	6–9	230	24694	0.93
18	9–12	152	16011	0.95
19	12–15	190	22814	0.83
20	15–18	184	23943	0.77

eriod some hours later partly depends n the oxygen content or the availability xygen to them.

have earlier also referred to my stud- on the effect of fractionated irradiation itrogen and air and mentioned that the ogen effect could be knocked out by a e of 1100 r in air. Lower dosages did have this effect.

rom this basis the problem arose ether or not there might be a recovery ells to which oxygen in air is available ot too low a concentration, i.e., sperm ing the first few hours after mating.

f there was a recovery process at work losages below 1100 r one would expect oportionally lower effect per r at lower ages and, in the dose range in which a overy began to be blocked, a proportion- higher effect per r which then at higher doses stabilized again. In other words one would expect a sigmoid curve (fig. 1) if there was a recovery process at work at a dose level below 1100 r. To study this possibility I made some experiments in which 3–4 day old males were irradiated and subsequently mated for 4 hours. They were kept without females the 4th–15th hour and were mated a second time, the 15th–18th hour after irradiation. The data from these experiments are presented in table 5 and in figure 2. From table 5 it is evident that already at the lowest dosage, 550 r, there was a significant difference between the two mating periods. In figure 2 the regression lines based on data in the dose range 550 r–3300 r are presented. There is a very good agreement with linearity in this range and the extrapolation to the control values co-

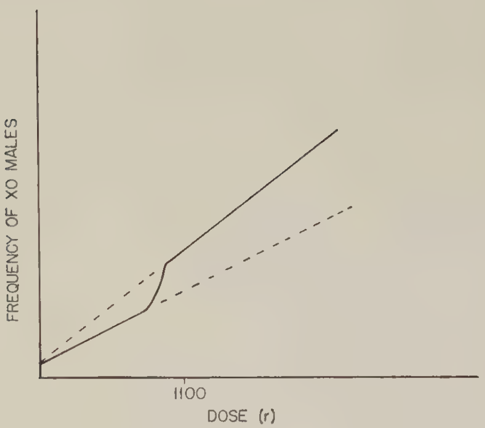

Fig. 1 The effect of a recovery process at work at low dosages but blocked at higher dosages.

incides rather well with that found in th control. We might conclude that this e periment has failed to show the occurren of a recovery process in sperm irradiate in a stage that is supposed to be rath well supplied with oxygen.

Although my last conclusion is rath negative as regards the recovery proce these experiments tend to show the nece sity of dealing with well defined cell stage as unknown differences in the techniq between series might give complete spurious results.

OPEN DISCUSSION

OSTER[1]: You seem to be relying mo on the integrity of your Drosophila male

TABLE 5

Mutation-dose relationship in sperm from 0–4 and 15–18 hours after irradiation

Treatment	Mating period					
	0–4 Hours			15–18 Hours		
	XO males	Total offspring	% XO males	XO males	Total offspring	% XO males
Control	55	80900	0.068			
550 r	177	80944	0.22	165	93752	0.18
825 r	104	37292	0.28	72	36347	0.20
1100 r	218	60130	0.36	177	63348	0.28
1650 r	317	65081	0.49	286	78921	0.36
2200 r	309	48690	0.63	309	58676	0.53
2750 r	210	25105	0.84	142	26997	0.53
3300 r	442	44528	0.99	462	62869	0.73
3850 r	200	14152	1.41	273	22976	1.19
4400 r	584	38649	1.51	413	35357	1.17

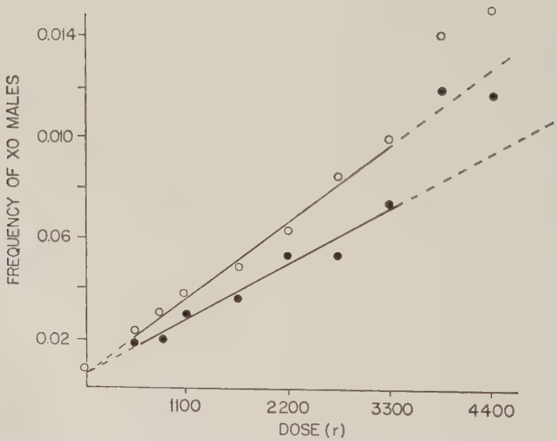

Fig. 2 The data from table 5. O, matings 0–4 hours, $Y = 0.000509 + 0.0007660 \times$. O, matings 15th–18th hour after exposure. $Y = 0.000539 + 0.0005507 \times$.

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[1] Irwin I. Oster, The Institute for Cancer R search, Philadelphia, Pennsylvania.

on the validity of your techniques,
se I gathered that you implicitly trust
of your males to mate with only one
e—in spite of the fact that you have
mass matings. I infer that your re-
are based on mass matings, are they

NING: Yes.

TER: How can you be sure that each
will be so "faithful" (if to no one
ut to the investigator) as to seek out
one female during the 4-hour period?

NING: This is of little importance
what we wanted to have was matings
n these first 4 hours.

TER: Then how would you explain
sults which was as well as others have
ned as regards this first and second-
mating? If you are careful and irradi-
e males on one day and mate them
idually to females, and then on the
d day separate them and give each
another female, and use only the
from matings which occurred on two
cutive days, you sometimes get a dif-
ce in the rate of whatever effect you
tudying, translocations or single-hit
s, between the first and second day
sometimes you do not. Such results
ate, do they not, that this seems to
rather variable phenomenon, unlike
one observes with other stages of
ophila?

NING: No. I think that the results
h I show in some of the papers where
nated the males once only showed a
good agreement with the time of the
ment. The result was a change at a
in period.

STER: I was referring to some data of
as well as some which Miss Nordback
ned while working with Dr. Auerbach.
Burdette showed data this afternoon
hich he didn't get it either. There is something else I want to ask. What were your control values for XO males? I think Dr. Abrahamson will verify they are sometimes appreciable. Using separate rates from separate mating periods—not a total—did you find the controls vary?

LÜNING: Dr. Savhagen's obtained the control values up to 11 days in her experiment and there were no variations at all.

ABRAHAMSON[2]: In this one experiment Dr. Oster refers to, our XO male frequency in the irradiated series was as high as the control. This was an unselected stock.

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[2] Seymour Abrahamson, University of Wisconsin, Madison.

Recovery in X-Irradiated Germ Cells[1]

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the following discussion I shall con-
nyself to a consideration of the evi-
for recovery following the X irradi-
of mature germ cells, since a number
her speakers have discussed mainly
nmature reproductive cells in which
evidence for the existence of repair
omena already exists (Russell *et al.*,
Oster *et al.*, '59). It might be assumed
Drosophila melanogaster would yield
derable information as to recovery
g place following X-ray treatment of
natozoa since one can employ with
organism the most refined methods
able today for assessing induced dam-
This expectation has not been justi-
s very little in the way of absolutely
tive data has been obtained for the
nce of recovery processes in mature
tes. Ironically, this paucity of posi-
evidence is in large measure due to
act that the major portion of radia-
effects on this organism can be readily
preted and ascribed to other causes
r than to actual recovery. The small
nt of evidence available *favoring* the
nce of recovery processes, however,
d not be overlooked but should be
ully evaluated on the chance that it
have an important bearing on our
rstanding of repair processes in gen-
In order to view the experimental
s to be described in their proper per-
ive three points should be kept in
:

When we speak of recovery or repair
ean a process or processes whereby
reversal of the initially induced dam-
takes place under some conditions
not under others.

Applying this definition, it follows
although the great majority of chro-
mes broken by X rays reunite in their
nal order (Muller, '53), the fact that
requency with which this occurs is related merely to the number of original breaks and to their spatial relationships rather than to the conditions existing during or following their production, does not qualify this nonspecifically conditioned rejoining of ends as a recovery phenomenon.

3. Several investigators have demonstrated (Auerbach, '54; Khishin, '55; Lüning, '52; and more recently by Oster, '59) that in addition to the differences in radiosensitivity between immature and mature germ cells tremendous differences in X-ray sensitivity exist among the latter types of cells. An example of the magnitude of variation in the sensitivity of the male germ line of Drosophila has been included from our previous paper and is shown on table 1 (Oster, '59).

These marked differences magnify the importance of treating and testing homogeneous samples of germ cells lest some shift in gamete utilization caused by changes in conditions either during or after treatment be erroneously interpreted in terms of recovery or some other phenomenon which happens to be in fashion at the time. In passing, I should like to point out that exceedingly accurate methods have been worked out for treating homogeneous samples of germ cells, particularly in the male (Auerbach, '54; Khishin, '55; Oster, '58). Large numbers of homogeneous samples of reproductive cells for treatment can be obtained by irradiating accurately determined stages of the life cycle that contain a preponderance of one type of germ cell and the utilization of only this one stage at a time for genetic analyses by the use of short mating periods following the maturation of the cells. Unfortunately only a relatively few investiga-

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[1] New observations here reported were made with the aid of a grant from the United States Atomic Energy Commission Contract AT(30-1)-2618.

TABLE 1

Results of X irradiation of different male cell stages in Drosophila

Cell stage	Dose	% Sex-linked lethal mutations	% Autosomal translocations
	r		
Untreated male germ cells	—	12/4,806 = 0.2	0/6,891 = 0
Spermatogonia (larvae)	2000	9/1,247 = 0.7	0/813 = 0
Spermatids (48-hour-old pupae)	1200	105/1,087 = 9.7	131/902 = 14.5
Spermatozoa (adult males) (released 1 day after treatment)	2800		62/905 = 6.9
Spermatozoa (adult males) (released 2 days after treatment)	2800		27/510 = 5.3
Spermatozoa (inseminated females)	2800		42/413 = 10.2

tors have made use of these methods thus far and the majority of workers are still clinging to the more antiquated and less reliable techniques.

Since our definition implies that recovery processes take place following the treatment let us consider the effects of subjecting X-irradiated spermatozoa to different types of treatment. The first observation that posttreatment can alter the mutation rate was made by Novitski in 1949. He found that exposure of spermatozoa which had been X irradiated in the seminal receptacles and spermathecae of females to −5°C. resulted in higher lethal mutation rates than when similarly X-irradiated sperm had been exposed to 25°C. This interesting observation suggests that the cold temperature may interfere with the functioning of the normal restorative mechanisms; further work along such lines is definitely indicated.

Subsequently, several investigators led by Baker *et al.* '52 (see review by Nordback and Auerbach, '56) have presented evidence that spermatozoa released on the first day following X irradiation yield a higher mutation rate than spermatozoa released on the second day following treatment regardless of whether or not the males supplying the second day's spermatozoa had mated on the first day. Most of this work involved studies of the frequencies of chromosome disarrangements, although similar results have been reported for recessive lethal mutations. Taken at face value, these results seemed to indicate that some recovery had taken place during the interval from the first
to the second day as a consequence
storing the spermatozoa in the male. Ho
ever, Lüning ('54) has suggested that ev
mature spermatozoa may differ in th
initial radiosensitivities in that those
leased right after treatment are more su
ceptible to having damage induced in the
than those released subsequently. To
count for the lower yield of mutations
spermatozoa released on the second d
after treatment even if the males had
mated on the first day he suggested th
resorption and ejaculation of unus
sperm normally takes place. Unfor
nately, the short-coming of this hypothe
lies in the fact that it is not yet know
whether or not such phenomena take pla
in Drosophila.

If we may assume for a moment th
Lüning's explanation is correct, then se
eral other related observations on irra
ated spermatozoa would be more read
understandable. For one thing, it w
noticed that although sperm released
the first day yield higher mutation rat
than those released on the second d
following treatment such results are
obtained with any degree of consistenc
Tables 2 and 3 illustrate some results
cently obtained in our laboratory which
show differences between the first a
second day sperm batches. But it must
pointed out that in other experiments c
ried out under ostensibly similar con
tions by us on other occasions such diffe
ences have not been observed (Oster, '59
The claim has often been made that t
age of the male is somehow related to t
extent to which the mutation rate of t

TABLE 2

esults of X irradiation of spermatozoa in the male

% Chromosome losses in sperm released on	
First day	Second day
6/1124 = 0.53	2/1524 = 0.13
8/979 = 0.82	8/1059 = 0.76
5/226 = 2.21	4/292 = 1.37

TABLE 3

esults of X irradiation of spermatozoa in the male

% Sex-linked lethals in sperm released on	
First day	Second day
55/1426 = 3.86	45/1886 = 2.39
91/1172 = 7.76	87/1442 = 6.03
54/338 = 15.98	44/430 = 10.23

and second-day sperm samples differ rdback and Auerbach, '56). In all of experiments, however, the age of the s was kept constant (i.e., they were –4 day old non-virgin males), so that factor could not have played a role ausing the variability in our results. only procedure in our experiments h differed from that of many other kers is that we always examined off- ng for genetical damage derived from le-pair matings in which the males mated on each of the two consecutive under investigation but this would tended to make for less rather than e variability. Such variability as we observed could in part easily have rigin in the fact that the amount of rption of unused spermatozoa which s place is rather variable.

order to account for the initial dif- nce in radiosensitivity we would like uggest that spermatozoa located in rent sections of the reproductive tract differently oxygenated under normal litions. It should be pointed out, how- , that the magnitude of this oxygena- is much less than that postulated by previously for the spermatid stage re an exceedingly large supply of oxy- must be present consistently in order to account for the high radiosensitivity of this stage (Oster, '58). In line with this view then, spermatozoa utilized on the first day following treatment would have been under somewhat a higher oxygen tension during the X-ray exposure than those cells released on the second day. This assumption appears logical since such spermatozoa, being closer to the genital orifice, would have better access to the surrounding air containing oxygen and therefore would be more sensitive to X irradiation than those farther back in the reproductive tract. It would also help to explain the variability of the results since under certain conditions such differential oxygenation might not occur. If this explanation coupled with Lüning's suggestion that unused sperm are resorbed constitute the bases for the observed differences, then the finding, based on extensive tests, that there is no difference in mutation rate between sperm released on the first day and those released on the second day following irradiation with fast neutrons could be readily explained. For with this type of radiation, it has been shown that the presence of oxygen during exposure plays a relatively slight role in enhancing the amount of damage induced (Gray, '59). Extending this argument further it may be postulated that the basis for the well-authenticated difference in sensitivity between spermatozoa X-irradiated while in the female and spermatozoa irradiated in the male lies in the fact that the former are relatively more oxygenated. That this difference in yield of mutations between spermatozoa irradiated in the male and those irradiated in the female is solely due to differences in radiosensitivity rather than recovery is suggested by the fact that storage of spermatozoa that had been X irradiated in the male for a fortnight or longer is without effect on the yield of mutations obtained. If this suggestion is correct, that the differences in mutation frequency among spermatozoa X-irradiated in the female and sperm X-irradiated in the male but released on subsequent days following treatment is due to differential oxygenation during treatment and hence to radiosensitivity rather than to recovery, then it would be expected

TABLE 4

Results of neutron-irradiation of different male cell stages in Drosophila

Cell stage	Dose	% Autosomal translocations
	rads	
Spermatozoa (adult male) (released 1 day after treatment)	750	29/725 = 4.0
Spermatozoa (adult males) (released 2 days after treatment)	750	41/872 = 4.7
Spermatozoa (inseminated females)	750	32/788 = 4.1

that they should be equally sensitive to neutrons or at least not show very great differences in their relative sensitivities. Attempts to verify this point have been undertaken and the results are shown in table 4. Since the rates are essentially equivalent it can be concluded that differential radiosensitivity is in all likelihood responsible for the differences which have been observed following X irradiation of spermatozoa. Needless to say, this conclusion does not preclude the existence of recovery processes in mature germ cells but it does indicate that additional work, perhaps following a somewhat different track, will have to be undertaken in order to demonstrate their presence.

OPEN DISCUSSION

Auerbach[2]: I must agree with Dr. Oster that there are some cases where one doesn't get the difference between first and second day. However, what matters is the interpretation of those experiments in which a difference is found. Two interpretations are possible. The first interpretation which Dr. Lüning favors, assumes there is a difference in intrinsic sensitivity between fully mature spermatozoa and almost mature spermatozoa. The second interpretation assumes that when fully mature spermatozoa are stored for 24 hours, in the male, there is a certain amount of recovery from mutation and chromosome breakage.

I think on both assumptions one can very easily imagine conditions under which this not very strong difference could be observed.

I want now to go to the second assumption. I have been interested mainly from the point of view of whether or not the first-second day difference is indeed due to recovery. At the time when this day difference was found, Dr. Russell hadn't yet obtained his evidence for dose-ra effects, and this seemed to be the first i stance where there was evidence of reco ery from genetic damage caused by X ray

It seems possible that there is a conne tion between the decrease in mutation fr quency from the first to the second da and the observation that irradiation mature spermatozoa in the inseminate female produces more mutations than irr diation in the male. There is a descendin order of the effects of a given dose of rays. High mutation frequencies are pr duced in inseminated females, next com the mutation frequency in spermatozo used by the treated males on the first da and last is that on the second day.

It seemed to me that if recovery is th explanation of the difference between fir and second day, the high mutation fr quency obtained by irradiation of insem nated females might simply be an expre sion of the fact that no recovery at a takes place in the female. In fact, recove appears to stop as soon as the sperm ha been transmitted to the female. So on may then imagine three situations: n recovery at all in inseminated females; certain amount of recovery in males du ing the first day; and full recovery in spe matozoa used on the second day.

I thought that one way of testing th would be to use neutrons, because it ha been shown by Dr. Lüning that there no difference between the first and secon day when neutrons are used. So the does not seem to be a recovery from ne tron damage between the first and secon day.

If the same applies to the differenc between inseminated females and treat males, then neutrons should not be mo

---

[2] Charlotte Auerbach, Institute of Animal netics, Edinburgh, Scotland.

ve when used on inseminated fe-
than when used on males.
Mary Alexander has just started to
is during a visit to Edinburgh. Mean-
this experiment has been done in-
dently by Dr. Oster.

AY[3]: The real test of your oxygen-
n hypothesis would be to do the
set of experiments in nitrogen. Then
vould know there was no influence
ygen. If each could be irradiated
anoxia, then you would obviously
nate this effect. Neutron experiments,
see, aren't conclusive on this point
se the neutron damage shows much
oxygen than X-ray damage depend-

TER: I believe that Dr. Baker carried
uch experiments in 1953 while he
at Oak Ridge and, indeed, he found
ifference in the amount of damage
een first and second day sperm when
rradiation was carried out in nitrogen.

NING[4]: I think that it might be an
en effect of the difference between
irst and second day, simply in that
second day sperm are more anoxic
for that reason there can be more
ery.

e opinion differs there between Dr.
bach and me as to whether they keep
perm or not.

N BORSTEL[5]: Is there any recovery
erm in the seminal receptacle over a
d of several days?

TER: No. We investigated that. We
iated sperm in the male and then
d the sperm in the female for 18 or
days. There was no appreciable
ge in the mutation frequency.

N BORSTEL: We found a similar ef-
We stored X-irradiated sperm in the
le and found no subsequent changes
duced mutation frequency.

BRAHAMSON[6]: There is one discrepant
t. If you irradiate the sperm in the
le, then hold it for over 18 days, they
an increase in translocation rate
18 or 20 days but no increase before
time.

TER: I made a special point of say-
sperm irradiated in the male since
aware of Herskowitz's results ob-
d by irradiating inseminated females
hich you no doubt are referring. Obviously, in his case, some indirect effect of the radiation on the females, perhaps mediated via the cytoplasm, must have played a role in enhancing the damage following the storage of sperm in their seminal receptacles and spermathacae.

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[3] L. H. Gray, Mt. Vernon Hospital, Northwood.

[4] K. G. Lüning, University of Stockholm.

[5] R. C. von Borstel, Oak Ridge National Laboratory.

[6] S. Abrahamson, University of Wisconsin.

chemical Processes in Injured Cells in Relation Cell Recovery

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e problem of cell recovery is probably ery different from that of growth and entiation. One can assume, as a ing hypothesis, that a cell or cellular m will recover from injury if the nor- processes leading to specific cellular ions still remain in working condi- In other words, at present nothing s to indicate the existence of special ms that will cause recovery. Perhaps njury will change the conditions so the cells that had stopped dividing enter a new phase of mitotic activity ll begin to form new specific constit- , but these processes need not, *a* , be different from those involved in al growth and differentiation. As we , these include: (1) the genetic sys- which, as discussed elsewhere in this osium, can be altered directly or in- tly by agents causing injury—even irect action may be reversible in some nces; (2) the specific systems that the information from the genes to the lar sites involved in division and dif- tiation, i.e., the ribonucleic acids A) and the protein-synthesizing sys- , often located in the cell cytoplasm; for this growth and differentiation to , the mechanisms of transfer of tic information from nucleus to cyto- n must remain functional, as well as ormal exchanges between the differ- cellular parts and between the cell and the outer environment; (4) for ese mechanisms to work, the cell will efficient phosphorylating systems.

e will not discuss all these mecha- s, which have been reviewed previ- , but we shall focus our attention on major problems, the ribonucleic acid protein-synthesizing mechanisms.

many cases, the injury is of very com- nature and can affect several of the systems referred to. This is the case with radiation which affects them more or less strongly and therefore makes an analysis of damage difficult. In this paper we aim chiefly to discuss some perhaps more radical or more specific treatments, that apparently have the virtue of leaving intact some of the mechanisms we have talked about. For instance, one can find conditions where it is hoped that *only the nucleoli* have been inactivated. This discussion will be concerned therefore with ribonucleic acid and protein metabolism in normal cells and in cells whose nucleoli have been inactivated by ultraviolet (UV) microbeam irradiations. A large number of these experiments were done by Dr. R. P. Perry with the collaboration of A. Hell. Their results led to the concept that in HeLa cells (and also in many other cell types studied by other investigators) ribonucleic acid synthesis takes place in the cell nucleus and from there migrates into the cytoplasm. This conclusion poses the problem of the *mechanism of the migration* and also whether the migrating nucleic acids undergo molecular reorganization during the process.

Work on this problem, using specific inhibitors, was started in collaboration with Dr. R. P. Perry and continued by Dr. P. Srinivasan in our laboratory.

MATERIAL AND METHODS

Most of the work was done with HeLa cells kindly supplied by Professor P. De Somer (University of Louvain) and Dr. D. Dekegel (Institut de Pasteur, Brussels) whom we thank heartily. They were grown on glass coverslips or in quartz cuvettes, incubated and microbeamed in the logarithmic phase as previously described in detail (Perry *et al.*, '61).

I. *Nuclear synthesis of ribonucleic acid*

In the present system, the demonstration of the nuclear origin of cytoplasmic RNA rests on two types of experiments, the kinetics of incorporation of the RNA precursors (H^3-cytidine and H^3-adenosine) into normal cells, and the study of the fate of cytoplasmic RNA metabolism in cells whose nucleoli have been inactivated with the UV microbeam.

Kinetics of riboside incorporation into nuclear and cytoplasmic RNA. The pattern of the incorporation of H^3-cytidine into HeLa cell RNA is shown in figure 1. The units of this graph are arbitrary and have been corrected for cell geometry and also for cytidine incorporated into DNA, measured after ribonuclease treatment at each time of observation. The figure clearly shows a striking difference between nucleus (nucleolus = n; the rest of the nucleus = N) and cytoplasm (= C). The rise is very rapid in the first case but only gradual in the second, and the incorporation rate seems to be zero in the cytoplasm at the beginning of the process. Furthermore, in the first case, the number of grains in N and n reach an equilibrium point after a few hours and increase steadily in the cytoplasm once it has started. Short incubation counts on N and n show the existence of a lag of a few minutes before the process starts simultaneously at comparable rates in N and n (fig. 2).

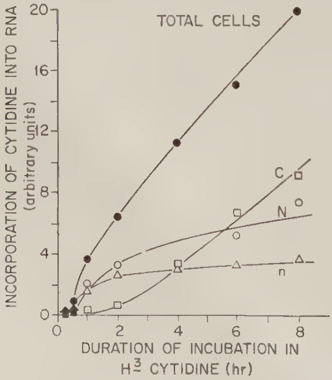


Fig. 1 Incorporation of H^3-cytidine into RNA in HeLa cells. Incubation mixture: media Φ 10 ± 0.4 μc H^3-cytidine (1.2 mc/mg). Ordinates: number of grains.

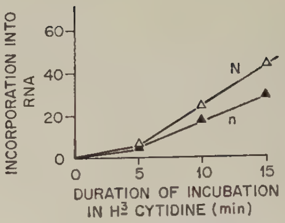


Fig. 2 Incorporation of H^3-cytidine into R
in HeLa cells same condition as figure 1. Or
nates: number of grains.

Effect of microbeam irradiation on cytidine incorporation. In these experiments the cells were grown, irradiated, incubat
and radioautographed in the same qua
cuvette made in an ordinary microsco
slide as described previously (Perry *et*
'61). The results of a typical experime
in which the cells were incubated for
hours in H^3-cytidine after having be
microbeamed are shown in table 1.

Table 1 shows: (1) that microbeami
a nucleolus inhibits drastically its capac
to incorporate the precursor (Col. I). T
conditions were such, that the irradiati
of one nucleolus did not affect the othe
of the same cell and the irradiation of
equivalent part of the nonnucleolar part
the nucleus did not affect to any great
tent the incorporation of cytidine in t
nucleoli; (2) that irradiating the nucle
of a cell inhibits to a greater extent t
cytidine incorporation in the other parts
the nucleus than does microbeaming ide
tical areas elsewhere in the nucleus (C
II). The difference between the activi
of a cell irradiated through N and a c
irradiated through n gives a value for t
nucleolar dependency of N RNA; (3) t
irradiation of the nucleoli of a cell inhib
more the cytidine incorporation in the cyt
plasm than does the microbeaming throu
N, a nonnucleolar part of a nucleus (C
III). Again the difference between the
two values will give the *nucleolar depen
ency of cytoplasmic RNA.*

If similar experiments are done usi
various times of incubation with the radi
active precursor, the results shown in tab
2 and the curves of figure 3 are obtaine
It can be seen that over 60% of incorpo
tion into cytoplasmic RNA is nucleolar
pendent, and that 30% of incorporati
into N RNA becomes nucleolar-depende
for incubation times exceeding 2 hou
These results are taken from observatio
of about 700 cells.

TABLE 1

Summary of experiment no. 61
Cells irradiated in designated areas and incubated 8 hours in H³-cytidine

I			II				III			
ea iated	Mean grains per nucleolus	Number of cells		Area irradiated	Mean grains per nucleus	Number of cells		Area irradiated	Mean grains per cytoplasm	Number of cells
e	6.5	28		None	41	28		None	43	28
ne	6.2	15	Nucleus	One	36	15		Nucleus	35	26
vo	4.8	12		Two	29	12				
			Nucleoli	some	26	8	Nucleoli	some	12	8
vo	1.1	20		all (two)	20	15		all (two)	5.5	14
hree	0.4	6		all (three)	10	6		all (three)	3	6

he following facts can be stated from
e two types of experiments: (1) RNA
ormed independently in n (probably
prising its associated chromatin) and
N. (2) After 2 hours incubation, 30%
the incorporation into N RNA is nu-
lar-dependent. (3) Sixty per cent of
incorporation into cytoplasmic RNA is
leolar-dependent. (4) The analysis of
kinetic curves for the cytoplasm, when
60% of nucleolar contribution has
n substracted, also show a zero rate at
beginning of incubation, indicating
t the rest of the cytoplasmic RNA me-
olism is *not* independent. Analysis of
kinetic curves led Perry ('60) to show
t this cytoplasmic RNA is dependent
N.

hese experiments therefore lead to the
owing picture: assuming that cytidine
ake by these growing cells represents
e RNA synthesis, one can state that
RNA is synthesized independently in the nucleolus (including its associated chromatin) and in the rest of the nucleus, and moves from these two sites into the cytoplasm.

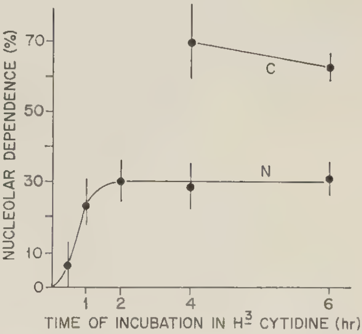


Fig. 3 Nucleolar dependence of N RNA (RNA from nonnucleolar part of the nucleus) and cytoplasmic RNA. The experimental points are calculated from table 2. (See text.)

TABLE 2

Tritiated cytidine activity after microbeam irradiation, expressed as the percentage of the average for unirradiated controls, together with expected error based on total grain count

Time of incubation in H³-cytidine	Nucleolar activity		Nuclear activity		Cytoplasmic activity	
	Nucleolus irradiated	Nucleus irradiated	Nucleolus irradiated	Nucleus irradiated	Nucleolus irradiated	Nucleus irradiated
hours	%	%	%	%	%	%
½	15±2	75±8	66±5	72±5		
1	10±2	91±9	54±4	78±6		
2	4±2	100±9	59±5	73±5		
4	10±2	100±9	59±5	88±6	45±5	115±10
8	13±2	78±6	50±3	81±4	22±2	85±4

All the cytidine taken up by the nucleus may not be incorporated into polynucleotide chains, for as pointed out by Vincent and Baltus ('60) the abnormally high cytidine incorporation found for starfish oocyte nucleoli could be partially due to end-chain cytidine labeling. In unpublished experiments Perry and Dürwald have also found that the nucleoli, relative to the rest of the nucleus (N), have a higher incorporation of cytidine than of adenosine.

The present experiments leave no place for significant independent cytoplasmic RNA synthesis, but give no precise information about the nature of the process of transfer of RNA from nucleus to cytoplasm nor about the nature of the RNA being transferred.

II. *Protein metabolism in HeLa cells*

Kinetic experiments. If a tritiated amino acid H^3 is incubated in a balanced salt solution with HeLa cells, it can be seen (fig. 4) that the kinetics of cytoplasmic incorporation is quite different from what is found with ribosides, and there is no lag in the uptake of these amino acids into the cytoplasm. Furthermore it has been shown elsewhere that the relative amounts of isotope incorporated into N and C are constant during 24 hours (ta 3) indicating that the incorporation

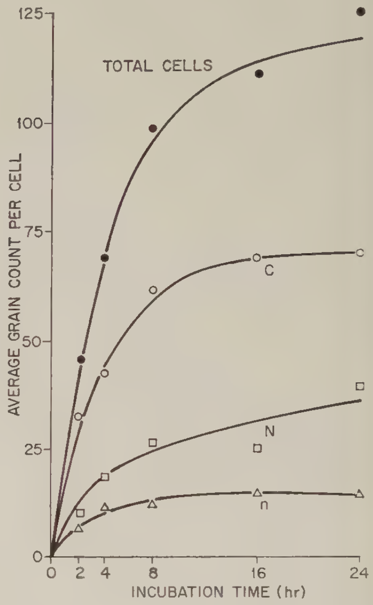


Fig. 4 Kinetics of H^3-lysine incorporation i HeLa cells. Incubation medium: balanced s solution (Hanks medium) containing H^3-lys (19 $\mu c/\mu mole$) at a concentration of 9 $\mu c/ml$.

TABLE 3

Per cent of total cell-grain count in different parts of the cell

Exp. no.	Incubation time	Nucleolus	Nucleus	Cytoplasm	Remarks
	hours				
9	½	36		64	
9	1	43		57	
21	2	7.8	31	61.2	Medium supplemented with amino acids
21	2	9.8	33	57.2	
29	2	9.0	32	59	
34	2	8.0	24	68	
65	2	7.7	34	58.3	
93	2	7.0	28	65	
34	2½	9.0	30	61	
64	2½	7.5	30.5	62	
21	4	8.3	35	56.7	Medium supplemented with amino acids
21	4	7.5	36	56.5	
29	4	11	41	48	
63	4				
79	4	5.0	34	61	
90	4	7.8	28	66	
62	4½	6.0	37	57	
66	6	7.0	33	60	
29	8	11.0	33	56	
34	11	8.0	31	61	
34	16	9.0	27	64	
84	16	3.0	31	66	
34	24	8.5	36	55.5	

TABLE 4

Effect of UV microbeam through nucleolus (n_{irr}) and "nucleus" (N_{irr}) on the incorporation of amino acids in different parts of the cell

Exp. no.	Incubation	No. of cells			Average counts in controls			% Activity remaining in n		% Activity remaining in N		% Activity remaining in C		
		Control	N_{irr}	n_{irr}	C	N	n	n_{irr}	N_{irr}	n_{irr}	N_{irr}	n_{irr}	N_{irr}	
Col. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	
	hr.													
64	2	30	24	27	69	34	8.3	80	107	109	110	97	98	Lysine
65	2	40	37	42	60	35	8	55	55	65	78	77	104	Lysine
93	2	50	33	33	22	13	2.9	98	99	111	104	97	98	Lysine
62	4	20	19	15	55	34	5.5	80	67	111	105	97	85	Lysine
79	4	16	12	14	31	17	2.8	88	76	71	87	71	87	Lysine
90	4	30	25	29	76	33	9.3	61	71	100	88	88	67	Lysine
66	6	20	20	20	42	23	4.7	69	100	86	100	56	94	Lysine
84	16	15	15	19	151	70	5.3	59	100	98	103	78	115	Lysine
31 32	2½	10	10	9	39	34	6.5	85	81	112	96	97	90	Phenylalanine
30 32	5	8	9	9		47	13	50	98	125	110			Phenylalanine

C, cytoplasm; N, nucleus without nucleolus; n, nucleolus.

these three cellular parts is probably independent (Errera *et al.*, '61). To obtain sufficient uptake of amino acid it has been necessary to use a balanced salt solution; one cannot therefore assume in these experiments, that one is dealing throughout with a true synthesis. However, at least during the initial incubation, it is probable that the pool of protein precursors is still sufficient for true synthesis to take place. The mitotic indices at 2, 8 and 24 hours have been counted and are still of 5.3, 4.1 and 2.5%. In the cytoplasm therefore this synthesis would be independent from the nucleus.

Microbeam experiments. Under conditions where the nucleolar RNA metabolism has been completely inhibited, the microbeam experiments already published (table 4) (Errera *et al.*, '61) have shown no effect on amino-acid uptake (lysine and phenylalanine) in any of the cell parts studied when the incubation was 4 hours or shorter. After that time (up to 16 hours) one finds that the nucleolar and cytoplasmic activity (not the N activity) drops by about 30%. This result indicates that the protein metabolism becomes dependent on RNA activity only after a few hours—and even after 16 hours we have found no evidence of nucleolar dependency of N protein metabolism. Therefore it is probable that the nucleolus has some remote control on cytoplasmic RNA metabolism since it takes more than 4 hours for nucleolar injury to become manifest and to reduce the cytoplasmic protein activity by
about 30%. Actually the amino-acid
take follows much more closely the R
concentration than the RNA metabolis
This is certainly true in N and n in wh
the ratio n/N of RNA concentrations me
ured by microspectrophotometry by Pe
(Perry *et al.*, '61) are approximately 0.
very close to what is found for lysine
take. The lysine uptake in the cytoplasm
lower than expected when cytoplas
RNA is measured by microspectrophoto
etry but close to what one would exp
from biochemical data on nucleo-cytopl
mic ratios (see table 5).

III. Nucleo-cytoplasmic transfer of RN

If the hypothesis that RNA is synth
sized in the nucleus (N + n) and th
transferred to the cytoplasm is correct,
should be possible to demonstrate th
transfer by labeling the cells so as to ha
high N + n activity (1 hour incubatio
for instance, see fig. 1) and then to f
ther incubate them in a medium conta
ing no radioactive precursor or even hi
amounts of the nonradioactive equivale
of the precursor used for the initial lab
ing. The cells are incubated for 1 hour
usual with the appropriate tritiated prec
sor (usually cytidine) in Eagle's mediu
and are thereafter removed to fresh Eagl
medium containing 100 times more of t
nonradioactive riboside. The cells are fix
after various times of "chasing" and t
grains counted as usual. In this mediu
there is usually a further rise in the tot
grain count indicating that the labeled p

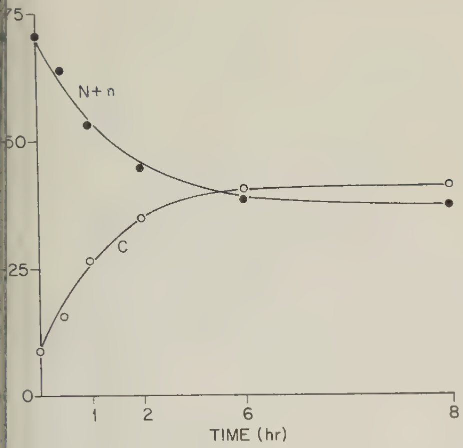
TABLE 5

Loss of acid insoluble nuclear C^{14}-adenine during homogenization of HeLa cells

Per cent of C^{14} tracks lost in bare nuclei compared to whole cells of the same homogenate[1]

	Time of incubation in C^{14}-adenine					
	10 min.	30 min.	1 hr.	2 hr.	4 hr.	17 hr.
	Experiment I					
Immed. after homogenization		20	22	10	4	
30 min. after homog. (at 0°C.)		33	44	21	11	8
	Experiment II					
Immed. after homogenization	30	29	28	23	10	
30 min. after homog. (at 0°C.)	36	38	35	36	15	

[1] The cells after incubation in a Roux flask with 0.45 μc 8-C^{14}-adenine/ml medium Φ10 are homogenized in sucrose $CaCl_2$ (Allfrey and Mirsky, '58) in a Virtis homogenizer (60% of maximum speed) until about 50% of the cells are broken up. The homogenate is smeared on microscope slide and radioautographed with Ilford G^5 emulsion according to Ficq ('57) after fixing in acetic alcohol and removal of acid soluble material in 2% PCA according to Perry *et al.* ('61).



ig. 5 Kinetics of transfer of cytidine incor-
ıted into RNA from nucleus to cytoplasm.
cissae time (hours) incubated in nonradio-
ve medium following 1 hour of H^3-cytidine
ake (0.9 μc of H^3-cytidine per ml of Eagle's
ium). Ordinates: number of grains in nu-
s (N + n) and cytoplasm (C) normalized to
e total as before transfer begins.

sor is not being diluted out by the high
centration of its nonradioactive coun-
part. However, if the total number of
ins after 1 hour in radioactive medium
l the various chasing periods are nor-
lized to an equal number, it can be seen
t the drop of radioactivity in the nu-
us equals the rise in the cytoplasm (fig.
therefore giving good evidence for nu-
o-cytoplasmic transfer of labeled mate-
. It has been noticed that the rate of
p of the radioactivity is initially greater
N than in n indicating perhaps that two
clear components are migrating, the
ter corresponding to N RNA, the slowest
haps to n RNA. More accurate data are
tainly needed to establish this point.

t is important to determine in what
lecular state the RNA thus formed in
nucleus, migrates into the cytoplasm,
l whether this transfer is done without
lecular rearrangement of the RNA.

everal types of experiments have been
ried out in order to obtain information
this matter. We have investigated: (1)
susceptibility to ribonuclease of the
idine-labeled RNA before or after its
nsfer to the cytoplasm; (2) the form of
nperature-dependency of this transfer;
) the dependency of this transfer on
h-energy phosphate sources.

Susceptibility of RNA to ribonuclease. One experiment, done in collaboration with Professor W. Plaut, showed that (1) after 1-hour incubation of HeLa cells in H^3-cytidine, the isotope is almost completely removed when the cells are further incubated at 37°C. for 1 hour in a medium Φ10 (Perry *et al.*, '61) containing 1 μg/ml of ribonuclease (Armour), although perhaps not quite as efficiently as when the cells are fixed; (2) the RNA is broken down and the low molecular weight compounds containing the labeled cytidine are not re-utilized if the cells are transferred after their incubation with ribonuclease into fresh nonradioactive medium; (3) if the labeled cells (1 hour in H^3-cytidine) are transferred to cold medium for 4 hours and only then submitted to ribonuclease at 37°C. both nuclear and cytoplasmic H^3-RNA are removed, indicating that even after prolonged incubation, the RNA that has taken up cytidine remains susceptible to ribonuclease after its transfer to the cytoplasm. As Brachet and Six ('59) have suggested for onion roots, this RNA might be chiefly of a non-sedimentable type.

Temperature dependency of RNA transfer. Transfer of RNA from the nucleus to the cytoplasm has been investigated at various temperatures between 20 and 40°C. in order to find out whether the pattern of temperature-dependency of RNA transfer and of RNA synthesis (uptake of H^3-cytidine) differed or not. The results are shown in figures 6 and 7. It can be seen that both uptake and transfer are completely inhibited at the lowest temper-

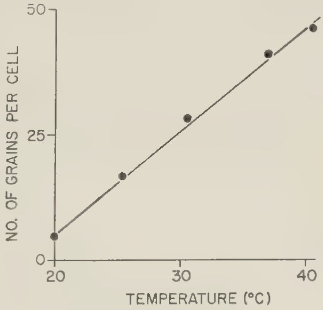


Fig. 6 Temperature dependence of H^3-cytidine uptake. Ordinates: number of grains per nucleus (N + n). Abscissae: temperature during incubation for 1 hour in 0.9 μc H^3-cytidine per ml Eagle's medium.

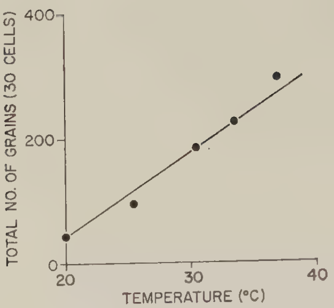


Fig. 7 Temperature dependence of transfer of H^3-cytidine incorporated in RNA from nucleus to cytoplasm. Abscissae: temperature during RNA transfer. Ordinates: Number of grains in the cytoplasm after 4 hours incubation in nonradioactive Eagle's solution containing 10 μgr cytidine of cells having incorporated H^3-cytidine for 1 hour.

atures studied, and the slopes of the temperature-dependency curves are roughly similar, so that no conclusion can be drawn from these experiments. It had been initially hoped that if a difference in temperature-dependency were found it might have been possible to distinguish an anabolic process from one where membrane phenomena or physical properties of the cell are probably involved.

Requirement for high-energy phosphate. Experiments done in the presence of high concentration of fluoride (10^{-2}M) or 2,4-dinitrophenol (5×10^{-3} M DNP) in Eagle's medium did not show any inhibition of *transfer*. In experiments with fluoride there was also no inhibition of *uptake* of cytidine after 1 or 4 hours incubation; in those with DNP although there was a striking inhibition at 1 hour, the cells had taken up as much cytidine after 4 hours as the controls.

Again these experiments have not led to a clear cut distinction between transfer and uptake, owing probably to the fact that in the case of DNP, at least, the expected inhibition of oxidative phosphorylation is compensated by a high glycolytic activity.

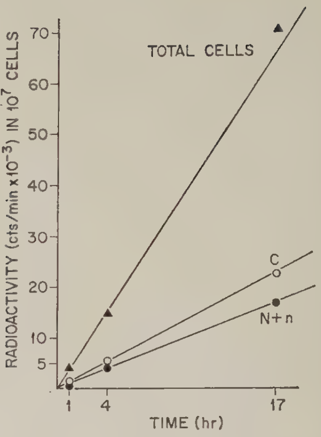


Fig. 8 Kinetics of adenine incorporation i HeLa cells and cell fractions. Abscissae: Ti of incubation in C^{14}-adenine (conditions: table 5). Ordinates: total count for 10 cells.

Preliminary biochemical analyses. Attempts to correlate radioautographic techniques with mass biochemical analyses have been made, in collaboration with Miss A. Faures, to obtain confirmation of the correctness of the cytochemical data and also to identify the state in which nuclear RNA reaches the cytoplasm. Inc porations of adenine-8-C^{14} were made Roux flasks. Consequently it was fou that the total uptake, like in the radi autographs, was linear at least for hours (fig. 8).

Fraction studies have, however, show that in the cytoplasmic fraction, the radi activity also increases almost linearly fro the beginning of incubation (although ad nine-8-C^{14} radioautographs show the san pattern as with H^3-cytidine) and the lev ing out of nuclear activity is not appare as it is in the radioautographs (fig. 8 This seems to indicate a *loss* of some n clear component during the fractionati procedures showing that for cells that hav incubated for short times, about 30% the nuclear activity is lost to the surroun ing medium and would be counted "cytoplasmic" (table 5). If the mean total activities recovered in nuclei an cytoplasm is compared to the mean of tot RNA recovered (table 6) it can be see that the loss of RNA during fractionatio is negligible compared to the loss of a tivity. This must indicate that a fractio of the order of a few % of the total RN but containing about 50% of the total a tivity, is lost during the fractionation. Pa of the adenine is therefore taken up into very labile and acid-insoluble componen which appears to be formed faster tha

TABLE 6

Total radioactivities and RNA recovered during fractionation of HeLa cells incubated with C^{14}-adenine[1]

Incubation time	% Radioactivity recovered compared to radioactivity of whole cells = 100			% RNA recovered compared to RNA of whole cells = 100		
	N	C	N + C	N	C	N + C
hours						
1	16	24	40	32	75	97
4	37	25	62	38	74	102
17	23	38	62	31	75	96

[1] These figures are the average of exp. 1–4 for the nucleus and 5–7 for the cytoplasm and are compared to results obtained on whole cells in companion experiments. RNA is determined according to Miller ('59); cell fractionations are done according to Joklick and Rodrick ('59); and chemical analyses as described by Faures and Errera ('61).
C, cytoplasm; N, nucleus without nucleolus.

component in which adenine is taken in a more stable form.

Nuclei are obtained from homogenates ere only 3–5% of cells remained intact l are purified according to Allfrey, wa, and Mirsky ('57). Cytological ob- vations on the nuclear fractions showed t although there were not more than 5% whole cells, there were approxi- tely still 10% of the nuclei containing ne of the cytoplasm attached to them l there were also some cytoplasmic umps" free in the suspension. This cyto- smic contamination can be determined m measurements of cytochrome oxidase itochondria) and glucose-6-phosphatase icrosomes). These measurements give otal contamination factor of 30% and % cytoplasm in these nuclear suspen- ns. When calculating nuclear RNA, s contamination can be corrected for m measurements of the amounts formed the same number of whole cells and in clei containing 25% cytoplasm contam- tion.

The cytoplasmic fractions are obtained m a homogenate where only 50% of ls are disrupted after spinning for 10 nutes at 10,000 *g* (F_1), for 90 minutes 100,000 *g* (F_2) and the supernatant m the last centrifugation is (F_3). F_1, F_2, l F_3 contain respectively 17, 27, 17% of total-cell RNA in fair agreement with figures given by Joklik and Rodrick 9).

The specific activities of the various ctions were then determined. The ac- ities of each fraction were compared to specific activity of whole cells taken as 0 and are tabulated in table 7.

These figures indicate that at short time incubations (1 hour) the specific activity of the nuclear RNA is only about 50% of that of total cell RNA, although the radioautographs have shown at this time that 80–90% of the labeled RNA is in the nucleus. This again means that a highly radioactive fraction of RNA is lost from the nucleus and explains the relatively high total "cytoplasmic" labeling of RNA in cells in which the radioautographic counts have shown it to be negligible. It also explains the linear cytoplasmic RNA activity on increasing incubation times. After 4 hours, the specific activity of nuclear RNA is about equal to that of total-cell RNA. It becomes smaller at 17 hours, a time when the radioautographs have shown that cytoplasmic activity is building up. These experiments also show that there certainly is a considerable loss of radioactivity during the fractionations, because the specific activity of total cell RNA is always the highest. The present results further indicate that after 1-hour incubation, the cytoplasmic fraction with the highest RNA activity is F_3, the supernatant of a 1.5 hours centrifugation at 100,000 *g*, it is therefore probable that this fraction at least, contains RNA coming from the nuclei which has been shown to be ribonuclease-sensitive. At longer incubation times, the heavier particles, which sediment in 10 minutes at 10,000 *g*, become the most active. It is difficult at present to interpret these results further before a safe correlation can be established between the fractions F_1, F_2, and F_3, and cytological entities such as mitochondria, microsomes, ribosomes, etc.

TABLE 7

Specific radioactivity of RNA of fractions[1] *of HeLa cells incubated for various times with C^{14}-adenine*

The figures are expressed as % of specific activity of RNA of whole cells (= 100)

Exp. no.	Time of incubation	Nuclei	% Total specific activity found in Cytoplasm			
			Total C	F_1	F_2	F_3
	hours					
1	1	47				
2	1	46				
3	1	51				
4	1	60				
6	1		22	16	25	39
7	1		40	38	15	43
1	4	107				
2	4	43				
3	4	97				
4	4	92				
5	4		33	19	27	41
6	4		28	21	16	30
7	4		52	51	60	72
1	17	71				
2	17	72				
3	17	66				
5	17		46	62	37	44
6	17		34	44	16	38
7	17		58	66	51	59

[1] Cytoplasmic fractionation is according to Joklick and Rodrick ('59) on a cytoplasmic fraction obtained after homogenizing to a point when about 40% of the cells were broken down and not more than 4% of the nuclei were disrupted.

However these data on "cytoplasmic" RNA can be compared to those of Frenster *et al.* ('61), who showed that nuclei having incubated for 60 minutes in radioactive orotic acid or adenosine, liberate in buffered calcium chloride, some particles containing ribonucleic acid that have incorporated orotic acid. When these particles are ultracentrifuged it is found that the smaller the particles, the higher is their ability to incorporate orotic acid and the supernatant RNA is one of the most active fractions. It can be shown also that the alanine incorporation ability of these particles *in vitro* is *not* affected by ribonuclease.

As we are certain from the radioautographic controls of the homogenized cells (table 5) that at 1-hour incubation, 30% of the adenine which has been incorporated in the nuclei passes into the "cytoplasm" during fractionation, we know that most of the radioactivity counted in the "cytoplasm" at 1 hour is of nuclear origin. It is therefore very probable that our fraction F_3 contains RNA related to the smallest and most active particles that can be isolated from the nucleus as ribonucleopr tein particles (Frenster *et al.*, '60).

DISCUSSION

Nucleo-cytoplasmic relationships in RNA and protein synthesis

Parts I and II of this paper are co cerned with the nuclear dependency (RNA and protein synthesis and the ge eral idea that there is a direct dependenc of cytoplasmic RNA on nuclear processe but only a remote dependency of cyt plasmic protein metabolism on the nu cleus. This is in complete agreement wit work by Brachet ('57); Prescott ('59 Zalokar ('60); Pelling ('59); and other Perry ('60) discussed the nuclear depend ency of cytoplasmic RNA metabolism i HeLa cells. The combination of kinet and microbeam experiments led him t conclude that in these cells, taken in th logarithmic phase of growth, almost all, not all, cytoplasmic RNA is under dire nuclear control. RNA precursors appear t be taken up independently in the nucleol (plus probably its associated chromatin

in the rest of the nucleus and from
e move to the cytoplasm. The nonnu-
lar part of the nucleus (N) would thus
tain two RNA's: one, on its way from
nucleolus to the cytoplasm which
unts to about one-third of N RNA, the
r two-thirds being synthesized directly
he chromatin and perhaps in the nu-
r sap. The cytoplasmic RNA would be
endent ± 65% on the nucleolus and
ut 30% on the rest of the nucleus (N).
n connection with this finding Edström
) has found a direct correlation be-
en the base composition of nucleolar
cytoplasmic RNA. Does this mean
in all conditions cytoplasmic RNA
abolism or synthesis is strictly depend-
on nuclear activity? It has been shown
rachet ('57) that anucleate fragments
cetabularia are still capable of taking
labeled RNA precursors, and chloro-
tic RNA synthesis goes on, even at a
her rate for several days after enuclea-
. This increased net synthesis is com-
sated for by a rapid drop of microsomal
nonsedimentable RNA that are much
e dependent on the nucleus. It is there-
possible that independent RNA syn-
is exists only in specialized systems
chloroplasts and perhaps also mito-
ndria and other more independent or-
elles.

Movement of RNA from nucleus to cytoplasm

he movement of RNA from nucleus to
plasm can very well be studied by the
ent technique of labeling and further
bation in cold medium. It can be
that the precursor taken up chiefly
the nucleus after 1-hour incubation
rates from there into the cytoplasm,
the experimental curves seem to indi-
that not all of the precursor incor-
ated leaves the nucleus. The cytidine
incorporated into the nuclear RNA
ery susceptible to ribonuclease *in vivo;*
mains susceptible to ribonuclease after
sfer to the cytoplasm. From the work
rachet and Six ('59) it is likely that
sedimentable RNA is the one most
itive to ribonuclease *in vivo.* The fact
in HeLa cells the RNA with the high-
specific activity after 1 and 4 hours
bation in radioactive adenine is nonsedimentable and sensitive to ribonuclease is in line with this concept. Part of this RNA could be "soluble RNA" (S RNA) which has the function of transferring activated amino acid to microsomal RNA; part might also be a carrier of genetic information into the cytoplasm; it is probably lighter than microsomal RNA and heavier than S RNA, and its high susceptibility to ribonuclease *in vivo* may be because it is not yet protected by a protein complement.

In an attempt to distinguish between RNA transfer and RNA synthesis we studied the temperature dependency of both these processes and found them to be rather similar. It has therefore not been possible to find temperature conditions when one of the two processes could be studied in the absence of the other. We also looked for conditions where synthesis and transfer of RNA could be distinguished by using inhibitors of phosphorylations. In the presence of high concentrations of fluoride (10^{-2} M) no inhibition of either process was observed when experiments were done in Eagle's medium. One experiment done in Φ10 did show a 50% drop in the uptake but not in the transfer of RNA, and this effect of fluoride might be due to the lower magnesium concentration of the Φ10. In the case of 2,4-dinitrophenol, we always found a normal transfer of RNA, but there was a very striking inhibition of uptake of cytidine after the first hour which was, however, usually compensated for after 4 hours. It is at present difficult to interpret these results in a manner that would throw some light on the mechanism of RNA transfer.

RNA synthesis and recovery processes

We have seen that in the present system there appears to be some dissociation between protein and RNA metabolism; whereas RNA metabolism of HeLa cells is exclusively and directly nuclear-dependent, protein metabolism is able to take place in the cytoplasm, independently of nucleolar metabolism—at least for some time. The present experiments have not demonstrated any dependency of cytoplasmic protein metabolism on the functioning of (N). The general significance of these experiments can probably best be discussed by comparing them to some of the data on enucleated organisms. Acetabularia are known to be able for some time to regenerate and to undergo morphogenesis in the absence of a nucleus. This process has been shown by Hämmerling to be under direct nuclear genetic control. The "information" once in the cytoplasm seems stable for some time and its expression is probably reflected by the high RNA metabolism found in normal and nonnucleated tips of the algae by Brachet and Olszewska ('60).

The nonnucleated part of the algae can still photosynthesize $C^{14}O_2$ into its proteins, increase its net amount of aldolase, fructosidase and phosphorylase for some weeks, though not synthesize the acid phosphatase of the nonsedimentable cytoplasmic fraction (see Naora *et al.*, '60; Brachet, '60).

Morphogenesis is in some way linked with marked sulfur metabolism. Methionine is actively taken up by the proteins of the nucleolus and also by the tip of the alga (Brachet and Olszewska, '60). Sulfur proteins play an important role in morphogenetic processes (Brachet, '59); reducing agents are able to inhibit them, not only in Acetabularia but in a variety of organisms like amphibian embryos and planarians. From a variety of experiments Brachet ('59) has postulated that the thiol-disulfide equilibrium in some manner controls the expression of the genetic information carried into the cytoplasm, and suggested that studies of agents capable of influencing the sulfhydryl-disulfide equilibrium on transferred RNA might be valuable.

There are a number of analogies and some dissimilarities between the HeLa cells which have been reported upon and the Acetabularia which we considered useful to discuss at some length. The HeLa cells are in logarithmic phase of division; the algae are growing and differentiating but not dividing during the time of study. However in both systems microsomal and nonsedimentable RNA are highly nuclear-dependent, and protein metabolism seems to some extent more remotely dependent upon the nucleus; the synthesis of some proteins is possible as long as the cytoplasm is not depleted of its RNA.

These experiments confirm those of Brachet and are in complete agreement
with present concepts that ribonuc
acids are intermediate information carri
from the genes to the place of specific
tein synthesis, although in the case
some proteins (perhaps the chromoso
proteins) there might be a more di
mechanism of information transfer.

These studies, if not dealing direc
with recovery, may be useful in approa
ing one of the fundamental proces
needed for all recovery processes.

OPEN DISCUSSION

Prescott[1] : I would like first of all
congratulate Dr. Errera and his collabo
tors. I think this work of Dr. Errera a
Dr. Perry is some of the best in settling
argument of whether or not there is a s
stantial and continuous transfer of ribo
cleic acid from the nucleus to the cy
plasm. Most people believe this occu
although perhaps it hasn't been prov
with complete conclusiveness. There
still a fair amount of resistance to the i
encountered from time to time among
chemists—particularly those who sugg
that some RNA fractions are synthesi
in the cytoplasm.

Our results, however, are in compl
agreement with Dr. Errera. In fact, we
an almost identical picture when meas
ing incorporation of a radioactive prec
sor into RNA. In the ciliated protozo
Tetrahymena exposed to tritiated cytidi
or uridine, nuclear RNA becomes label
first. The nucleus of this cell has a t
mendous capacity for synthesizing RN
The cytoplasm of *Tetrahymena* cons
tently shows a delay in the labeling of
RNA, and, to limited extent, we can
perimentally change the length of this
lay. Eventually, however, the cytopla
exceeds the nucleus in the amount
labeled RNA. This type of relationship
tween the labeling of nuclear and cy
plasmic RNA is consistent with the id
that the nucleus is the exclusive source
RNA. These comments on *Tetrahyme*
refer to the macronucleus. The micron
cleus is so small that detection of RN
synthesis within it would be very difficu

These experiments involve continuo
labeling with uninterrupted presence
the radioactive precursor. In a pulse

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[1] D. M. Prescott, Oak Ridge National Laborat

iment with removal of the label from medium at a point before the cyto- sm begins to show any labeled RNA, the leus continues to increase its radioac- ty for a time after this removal because a large pool of RNA precursors that can- be washed out of the living cell. The leus then gradually relinquishes its ioactive RNA to the cytoplasm. When nucleus is emptied of radioactive RNA, cytoplasmic radioactivity no longer in- ases. Again, this supports the idea that A in the cytoplasm is derived from the leus.

The question remains whether *all* the A in the cytoplasm comes from the nu- us or whether the cytoplasm has an in- pendent capacity to make this macro- lecule. We have done another type of periment on a number of cells—namely, cut cells into nucleated and enucleated ces and to examine the abilities of these gments to perform various activities. ucleated *Tetrahymena* fragments live 24 to 72 hours. In the case of amoeba, h fragments may survive for 2 or weeks. When *Tetrahymena* fragments immersed in nutrient media con- ning tritiated uridine or cytidine, one ds that the enucleates are never able to orporate any radioactivity into RNA. cleated fragments incorporate radioac- ity at a rapid rate. This is an absolutely an-cut black and white result. Enu- ated pieces of cytoplasm do not make A.

If the same experiment is repeated with adioactive amino acid, for example with tiated histidine, protein synthesis can be monstrated in enucleates, but it takes ace at a much lower rate than in nu- ated fragments. We have correlated the ount of RNA in the enucleated cell with ability to incorporate amino acids into otein and found the following picture in *trahymena*. During the first 12 to 24 urs, the amount of RNA in the enucleate es down precipitously. The capacity of ch cells to incorporate tritiated histidine o protein declines rapidly and is com- tely lost by 8 to 12 hours after enuclea- n. By this time the fragments have lost average of 30% to 40% of their RNA. suggests that there may be a fraction of A that is continuously supplied by the nucleus normally and is used up during protein synthesis. When this fraction is exhausted in enucleated cells, amino acid incorporation stops.

KELLY[2] : Dr. Prescott, I think we must remember that almost all the biochemical studies which have suggested independent synthesis of RNA in the cytoplasm have been done in liver. These are not dividing cells, in contrast to most of the cells which you and Dr. Errera have studied. They have a high intracellular turnover of RNA and protein and may well have additional cytoplasmic synthetic pathways. I do not think the kinetic studies by Barnum, Potter, and many others can be ignored. While the autoradiographic experiments clearly demonstrate transfer of nuclear RNA to cytoplasm I do not see how they can rule out independent cytoplasmic synthesis at this time.

Dr. Errera, have you done similar studies with adenine so we can compare them with the earlier studies of Logan and yourself? Also have you tried X rays on your cytoplasmic RNA?

ERRERA: We did do some studies with adenosine with identical results. Adenosine is taken up in the nucleus and moves to the cytoplasm.

As far as the cytoplasmic RNA is concerned, one must be very cautious because the mere fact of homogenizing cells may create artifacts: we have mentioned that after short incubations, a small but very radioactive nuclear RNA is lost to the "cytoplasm" upon homogenization. This small fraction could very well pick up RNA precursors when outside the nucleus.

If we can come back to the radiation work, I have done only one experiment and that is why I didn't talk about it. I will give the details of this experiment which we have to repeat.

We gave cytidine for 1 hour. Then we irradiated with 100, 300, or 900 r in different experiments, after that we further incubated the cells for 4 hours in non-labeled medium.

We see two things in this experiment. The total number of grains decreases: after 100 r we find only about 80% of the grains; after 300 r, about 60% ; after 900 r, about 40%. This result seems to indicate that this RNA or this material in which the cytidine is taken up is highly labile both toward X rays and ribonuclease.

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[2] Lola S. Kelly, University of California, Berkeley.

If we measure in the radioaudiographs, the ratio grains in nucleus/grains in cytoplasm, we see that after 4 hours the ratio is the same whatever the dose and that the cytoplasmic grains have increased during the 4 hours in the cold medium; this seems to indicate that what is sensitive is the newly formed RNA or at least the molecule in which the cytidine is taken up.

The mechanism transfer from nucleus to cytoplasm does not seem sensitive to X rays. That is how we interpret these preliminary results.[3]

NYGAARD[4]: This is a comment in connection with Dr. Kelly's remark. With respect to Barnum's curves (Arch. Biochem., 29: 7, 1950), they definitely show that the RNA of the nucleus has a very high turnover. However, in view of present knowledge that cytoplasmic RNA is far from a homogeneous mixture, I think we should realize that the incorporation we find in the "supernatant" RNA from a non-specific precursor such as radioactive inorganic phosphate might be due to the labeling of the "transfer RNA" (S-RNA) and consequently one cannot expect this fraction to satisfy the calculations of the precursor-product relationship. Also, I believe we are today willing to accept that there are at least two separate metabolic fractions of nuclear RNA. If we want to establish a precursor-product relationship based on "kinetic" curves I think that we ought to be very sure that we are comparing the pertinent curves and not curves representing heterogeneous mixtures of RNA.

So I would say I don't think that you can really apply those data (by Barnum) to the present case. I think another complicating factor is the very high rate of protein synthesis in the liver cytoplasm with its concomitant rapid turnover of "transfer RNA" and this, of course, will obscure any relationship between nuclear RNA and another supernatant RNA with a lower level of incorporation. I believe we cannot by any means discount the data that the people working with autoradiography are presenting. On the other hand, I think their interpretation of total grain counts
should be regarded with the same amou
of caution, because they, as well as
biochemists, are not always sure that th
are studying one single fraction of RN

ERRERA: Of course, I must be hon
about these results. Actually the calcu
tions show that we cannot account
100% of the cytoplasmic RNA comi
from the nucleus; the data are not th
accurate. I think we have to leave a 10
uncertainty factor which might leave pla
for independent cytoplasmic RNA form
tion. But at present there is no real e
dence for this.

PRESCOTT: Dr. Kelly, were those expe
ments from Barnum's laboratory on
mogenized tissues?

KELLY: The incorporation was *in vi*
You have to homogenize them separate

PRESCOTT: I would like to add to
Nygaard's notes of caution. This is, if y
take a nucleus out of a cell it becomes
reversibly damaged almost immediate
Anyone who has ever tried to make n
clear transplantations and to obtain s
vival of the transplanted nucleus is w
aware of the tremendous lability of t
nucleus. This means that the nucleus
altered by removal from its normal cy
plasmic environment. With respect
this, there have been several papers
cently, and we have checked the point ou
selves, which report that when isolat
nuclei are prepared from homogenates,
substantial leakage of RNA from the n
clei occurs within the first few minut
after isolation. Some of this work h
been done on liver nuclei. Any conclusio
drawn from measurements of specific a
tivities or base ratios of RNA derived fro
the various homogenate fractions are ope
to very serious doubt.

I think we are again confronted with th
uncertainty principle and will have to p
all of the biological and biochemical da
together in order to come up with the be
single conclusion.

UPTON[5]: With reference to comparati
studies of UV irradiation of nucleoli
different kinds of cell, I should like to a
if the effects on the nucleolus appear to

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[3] This experiment has since been repeated a
confirmed.

[4] Oddvar Nygaard, Western Reserve Universi

[5] A. C. Upton, Oak Ridge National Laborato

or none in each instance, and whether
relative radiosensitivity to UV varies
different kinds of organisms.

ERRERA: In these experiments, of course,
ere we are using the UV microbeam, we
using very high energies ($\pm 8 \times 10^{-3}$
s/μ^2/sec.). I think we are not studying
y specific UV effect, it would be just the
ne as if we took any unspecific dena-
ing agent.

f one looks at radioautographs after X
s, I don't think one can say specifically
t the nucleolus is more sensitive than
rest of the nucleus. We did think this
s the case with UV irradiated starfish
ytes a few years ago.

NOVELLI[6] : I would like to make a bio-
emical comment with respect to the in-
pretations of grain counts as reflecting
uclear as opposed to a cytoplasmic syn-
sis of RNA. It is clear now that at least
ee nucleotides can be added to transfer
A in a reaction that is independent of
A. All transfer RNA's have a cytidine,
idine, adenine terminus that seems to
on and off quite easily. Thus this end
the RNA could conceivably become la-
ed in the absence of nuclear participa-
n. On the other hand, Weiss, Hurwitz
d Andrey Stevens have demonstrated
te convincingly that another type of
A is synthesized in a DNA-dependent
ction. This RNA could very likely be
thesized in the nucleus. Perhaps some
the conflicting observations being dis-
ssed would be resolvable on the basis
t one is looking at two different kinds of
A with different origins and functions.

ALEXANDER[7] : I would like to take issue
h Dr. Prescott, who says we have got to
me up with the best single solution, be-
use I think this is the very thing we
stn't do.

There are marked differences in the
oplasmic structure between the cells
t are differentiated and produce pro-
n for functional purposes, and the cells
t are only concerned with reproduction.

In a recent note in Nature, two col-
gues of mine, Mercer and Burbank,
mmarized the tremendous differences
structure; and the electron microscopists
uld certainly conclude that there might
quite different mechanisms in cells that
ve to make protein and RNA merely for
their own reproduction, and those that have to have a machinery for making protein for functional purposes.

VON BORSTEL[8] : For the benefit of those who irradiate mammalian cells, an interesting relation just occurred to me which derives from Dr. Goldstein's experiments on HeLa cells (Exptl. Cell Research, *19:* 322, 1960).

Goldstein and his collaborators found that the enucleate half of a cell would live a lot longer, or at least appear viable a lot longer, if it were in conjunction or very near a nucleate cell, indicating that there may be some transfer of material either to get rid of waste products from the enucleate cell or to pick up some viability factor from the nucleate cell.

This may be relevant to Dr. Elkind's work, where irradiated mammalian cells seem to vary in sensitivity relative to their proximity with other cells.

Perhaps Dr. Prescott could present some information here based on his work with protozoa.

PRESCOTT: At one time we tried to find out whether or not an isolated enucleated amoeba would survive longer than a large group cultured together. Large groups of enucleated cells did survive a little longer than isolated enucleates. We went to the next logical experiment and put nucleated cells with enucleates, expecting to obtain even better survival of enucleates. The effect was just the opposite; when nucleates were present in the culture, the enucleates cytolyzed sooner. At that point we gave it up as a bad experiment.

POWERS[9] : In connection with the last subject, I thought that there was a number of observations in the literature which showed that, when there were large chromosome aberrations which might be lethal if the cell were by itself, it was not noticeably important if the cell were in contact with other cells with complete genomes.

I want to read into the record our observations that Dr. Ehret and I observed in *Paramecium bursaria* a number of years ago that nucleolar bodies are extruded from the macronucleus into the cytoplasm (Exptl. Cell Research *9:* 241, 1955).

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e Effect of Ultraviolet Light and X Rays on an nzyme-Forming System

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The problem of how living cells syn- esize specific proteins from free amino ids is one that is central to all of biology. esumably the number and kinds of pro- ns that a cell can make is specified by genetic content of that cell. Recent vestigations make it likely that the in- rmation governing the specific sequence amino acids in a given protein is con- ned in the gene. The study of protein nthesis in cell-free systems has been tively investigated by biochemists for past 15 years. These studies have dealt gely with the incorporation of C^{14}- beled amino acids into protein and have oduced results that permit a general out- e of a possible step-wise conversion of nino acids into peptide bonds. The acti- tion of amino acids was reviewed at evious Biology Division research con- ences in 1957 by Novelli and DeMoss d, in 1959 by Lipmann who reviewed me subsequent steps. The entire process the incorporation of amino acids into otein has recently been reviewed in de- il (Zamecnik, '60). Therefore, we need ly to consider the current outline of the eps involved.

Scheme of incorporation of amino acids into protein

$$E + ATP + aa\text{-}C^{14} \rightleftarrows E\text{-}AMP\text{-}aa\text{-}C^{14} + PP$$

$$E\text{-}AMP\text{-}aa\text{-}C^{14}S\text{-}RNA \rightleftarrows S\text{-}RNA\text{-}aa\text{-}C^{14}\ AMP + E$$

$$S\text{-}RNA\text{-}aa\text{-}C^{14} + RNP + E_2 + GTP \rightarrow RNP\text{-}PrC^{14}$$

In the first step amino acids are “acti- ted” by reaction with adenosine triphos- ate (ATP) leading to the formation of enzyme-bound amino acyl adenylate. ere seems to be a different enzyme for e activation of each amino acid. In a sec- d step the enzyme-bound amino acyl enylate transfers the activated amino id to transfer RNA (S-RNA). Here again ere seems to be a specific RNA for each of the amino acids. The amino acid is carried on the ribose of the terminal adenosine moiety. All transfer RNA's terminate in the sequence -C-C-A; therefore the amino acid specificity is resident in some other portion of the nucleotide sequence. In a final, complicated, and as yet poorly understood step, the amino acid is incorporated into protein on the surface of a ribonucleoprotein particle (RNP) called a ribosome. This final step requires guanosine triphosphate (GTP) and at least one enzyme that seems to be species specific (Nathans and Lipmann, '61).

There are several shortcomings in studying protein synthesis by observing the incorporation of C^{14}-labeled amino acids into protein. (1) In general one simply measures the radioactivity of protein precipitable by trichloracetic acid (TCA) rather than the synthesis of a specific protein, except in the study of hemoglobin synthesis. (2) The incorporation of a given labeled amino acid is usually not influenced by the addition of the other amino acids. (3) The incorporation has a very short time course and the incorporated amino acid remains associated with RNP, little if any radioactive soluble protein being formed and (4) this scheme does not provide for any role for deoxyribonucleic acid (DNA) (direct or indirect) in protein synthesis. For these reasons we decided some time ago to attempt to develop a cell-free system that would synthesize a specific protein.

Studies on ornithine transcarbamylase. In setting out to develop a cell-free system that will synthesize a specific protein, certain features and desirable properties of the system can be used in making a choice.

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[1] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

(1) The protein to be synthesized should be one that is easily, quickly, and accurately measured. These features suggest that the protein should be an enzyme. (2) The source of material for the preparation of the cell-free system should be readily available and reproducible. A variety of source materials have these features, but because of their rapid growth (and rapid protein synthesis), bacteria seem to be a very good source of material. (3) The enzyme selected for study should be one that is easily purified and one that is antigenic, so that specific antisera could be used to isolate small quantities of the enzyme from reaction mixtures. (4) Ideally it would be desirable if initially there was an absence or very low level of enzyme present in the system and whose synthesis could be initiated by the investigator. All of the foregoing properties suggest that a substrate inducible enzyme would have the best chance of yielding an active preparation. In 1957, however, we wished to avoid the inducible enzymes fearing that we might become involved in a study of the phenomenon of induction (poorly understood at that time) rather than to study protein synthesis. Subsequent events have shown that this was not a justified assumption, and we did indeed eventually return to the study of an induced enzyme.

The system that Palmer Rogers and I decided to explore is the synthesis of ornithine transcarbamylase in *Escherichia coli.* This enzyme is involved in the biosynthesis of the amino acid arginine. The pathway of synthesis of arginine in *Escherichia coli* is shown in figure 1. It starts with glutamic acid which is converted in turn to acetylglutamic, acetylglutamic semialdehyde, acetylornithine, ornithine, citrulline, argininosuccinate, and finally arginine. The enzyme ornithine transcarbamylase (OTC) catalyzes the conversion of ornithine to citrulline. Gorini and Maas ('57) discovered that the synthesis of this enzyme is regulated by the level o arginine in the culture medium. Hig levels of arginine repressed the synthesis of enzyme and low levels or the absence o arginine de-repressed the synthetic machinery so that an explosive synthesis o enzyme occurred. In fact, the entire pathway of arginine biosynthesis in this organism is regulated by arginine (se Vogel, '61).

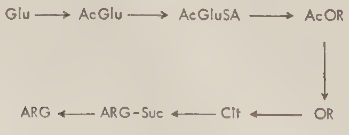


Fig. 1 Pathway of the biosynthesis of arginine by *E. coli.*

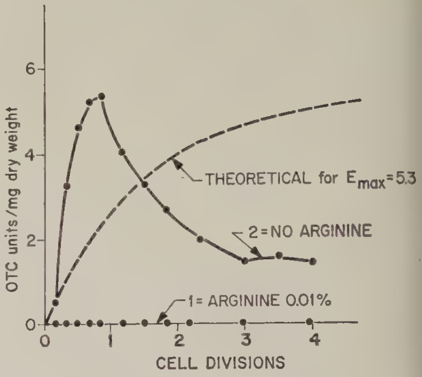


Fig. 2 Kinetics of OTC synthesis by *E. co* w (adapted from Gorini and Maas, '57).

Figure 2 shows some data from Gorin and Maas. The cells were grown in a medium containing arginine so there was relatively low level of OTC in the cells During logarithmic growth the cells wer removed from the medium, washed, an resuspended in the same medium in th absence of arginine. A rapid synthesis o OTC occurred that reached its peak jus before the first cell division. At this poin the entire pathway of arginine synthesis was de-repressed and internal arginine then accumulated and repressed further enzyme synthesis. The total enzyme molecules in the population remained constant but were distributed among the progeny. It was this burst of enzyme synthesis following de-repression that prompted ou investigation of this system.

Table 1 shows the reaction and how i is measured. We studied the synthesis o OTC by cells and protoplasts and obtained the usual evidence suggesting *de novo* enzyme synthesis (Rogers and Novelli, '59). Since these results have been published will not dwell on details but rather make one point that will be pertinent to our subsequent discussion. During this study w

TABLE 1

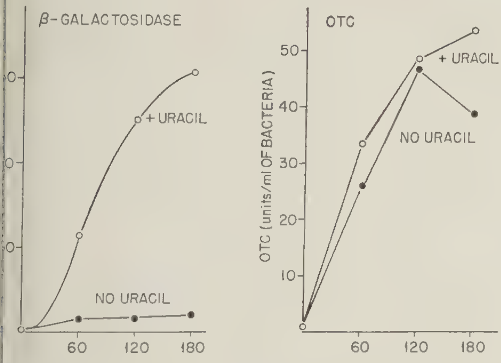
Ornithine transcarbamylase

ction:
L-ornithine+carbamyl $PO_4 \overset{OTC}{\rightleftarrows}$ citrulline+PO_4

y conditions:
Total volume — 1.0 ml
100 μmoles Tris pH 8.5
5 μmoles L-ornithine
5 μmoles carbamyl PO_4
Enzyme or toluenized cells (25–100 μg protein)
H_2O to 1.0 ml

Incubate 10–15 minutes at 37°C.
1 enzyme unit = 1 μmole citrulline formed per hour

iced that synthesis of OTC in protosts was inhibited by ribonuclease. In ole cells, enzyme synthesis was inited about 30% by 6-mercaptopurine, t high concentrations of 8-azaguanine or romouracil had little or no effect. These ults suggested that perhaps a nucleic d component might be involved in the thesis of this enzyme. This idea was ted with a uracil-requiring mutant. The ults of this experiment are shown in ure 3. In this experiment the synthesis OTC and of the induced enzyme β-galacidase are compared. The cells were wn in the presence of both uracil and inine and in the absence of an inducer β-galactosidase. The cells were harted, washed, and starved for an hour order to exhaust endogenous uracil. e experiment was started by placing the ls in a medium lacking arginine (to gger synthesis of OTC) and containing an inducer thiomethyl-β-D-galactopyranoside (TMG) for β-galactosidase. The data show rather clearly that β-galactosidase is not made in the absence of uracil, but that synthesis of OTC proceeds essentially normally in the absence of uracil. One straightforward interpretation of these results is that for the synthesis of β-galactosidase to occur a simultaneous synthesis of RNA is necessary, whereas OTC synthesis can proceed in the absence of RNA synthesis. This interpretation can be extended to current hypotheses of protein synthesis that argue that the protein-forming templates contain specific RNA. Thus for the case of OTC the data are consistent with the idea that during repressed growth on arginine the RNA templates are indeed synthesized but are prevented from making enzyme owing to the presence of the repressor. Upon removal of arginine the fully developed, enzyme-synthesizing machinery is capable of immediate activity thereby accounting for the explosive synthesis. For β-galactosidase, however, the data would suggest that during growth in the absence of inducer, the RNA templates are not made (i.e., the system is repressed further back) and when inducer is admitted to the system the RNA templates must be made before enzyme synthesis can occur accounting for the requirement for simultaneous RNA synthesis. With this interpretation the main difference between these two enzyme-forming systems would be that the β-galactosidase system is repressed at the level of DNA (the information template) and the OTC system is repressed at the enzyme-forming template. Alternate interpretations of these data are possible and I will return to this discussion later.



ig. 3 Synthesis of β-galactosidase by uracil- *E. coli* mutant (from Rogers and Novelli, '59).

We continued our studies of this enzyme and eventually achieved the cell-free synthesis of OTC by disrupting protoplasts in a Hughes Press (Rogers and Novelli, '60). Representative data are shown in table 2. Protoplasts, disrupted in a Hughes Press, can be centrifuged to yield a particle and a supernatant fraction. The active component of the supernatant can be precipitated by lowering the pH to 5.0. Neither fraction alone has any activity but when washed particles and the pH 5.0 fraction are combined there is an increase in en-

TABLE 2

Cell-free synthesis of OTC

Material	Control	Δ Enzyme units	
		ATP	ATP, UTP, CTP, GTP
Disrupted protoplasts	3.2	15.0	16.0
Washed particles, 105,000 × *g*	2.6	1.2	2.6
pH 5 fraction	0	0	0
Particles + pH 5 fraction	−1.8	5.0	9.2
Aged particles + pH fraction	−1.8	−0.6	0.4

Adapted from Rogers and Novelli ('60).

zyme activity upon incubation at 37° that is dependent upon a source of energy and the 4 nucleoside triphosphates. It would seem that new synthesis of enzyme had indeed occurred in this cell-free system. A more critical criterion for *de novo* enzyme synthesis would be to repeat the experiment with radioactive amino acids, isolate the enzyme at the end of the incubation period, and show that it was radioactive. This enzyme had not been purified from *E. coli,* and specific antiserum was not available. These experiments were halted in order to purify the enzyme and make specific antiserum so that this critical test could be made. I understand that Dr. P. Rogers has been successful in preparing antiserum to OTC (Rogers, personal communication). It should be possible now to carry out a definitive experiment.

Induction of β-galactosidase: inhibition by UV irradiation and photoreactivation. While the foregoing experiments were in progress, Dr. T. Kameyama joined our group and influenced us to begin an investigation of the effect of UV light on
coli. Kameyama and Suzuki ('60) ha
made an interesting observation of th
effect of exposure of *E. coli* to UV on
ribonucleoprotein component of the cel
An example of their data can be seen i
figure 4. Here cells were exposed to leth
amounts of UV and then incubated for 1
minutes in growth medium supplemen
with P^{32} after which the cells were broke
open and the crude extracts subjected t
electrophoresis on starch. The most stri
ing result, seen in figure 4, is the loss
peak II after exposure of cells to UV
Kameyama was anxious to follow up thi
observation, and, since most of the work
our laboratory was concerned with protei
synthesis, we decided to attempt to corr
late the loss of this ribonucleoprotein com
ponent with some aspect of protein syn
thesis. For this purpose we elected to stud
the induced synthesis of β-galactosidase
since it was known from other system
that the induction of some enzymes is sen
sitive to UV (Goucher *et al.*, '55; Swenso

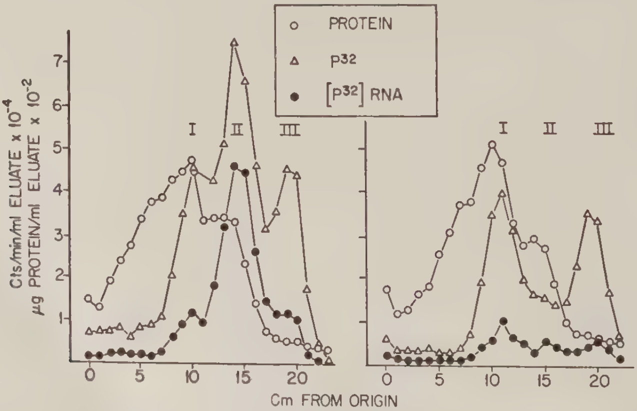


Fig. 4 Electrophoretic patterns of normal and UV treated *E. coli* (after Kameyama and Suzuki, '59).

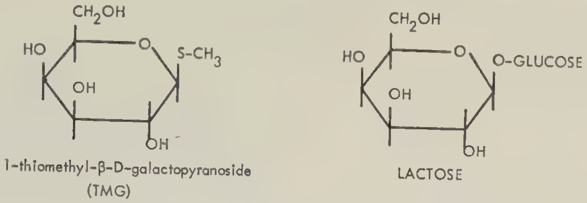


Fig. 5 Structure of inducer.

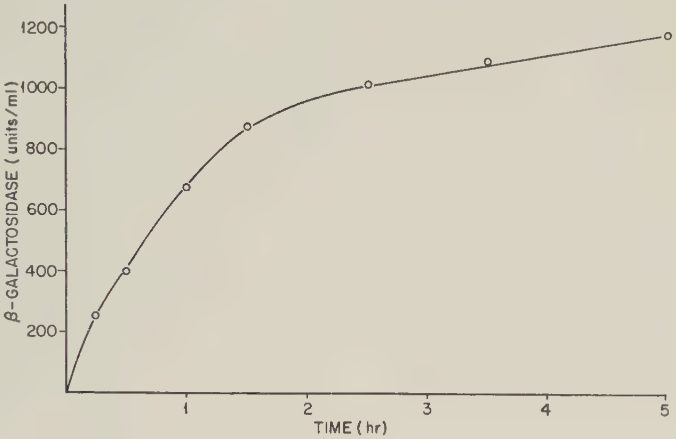


Fig. 6 Kinetics of β-galactosidase induction.

Giese, '50; Brandt *et al.*, '51; Kaplan
l., '53; Torriani, '56; Monod, '56, '58;
Cohn, '57). β-Galactosidase is a well-
wn enzyme, having been extensively
estigated by Monod and Cohn and their
workers (Monod, '56; Cohn, '57). It
the nice feature of being induced not
y by its substrate, lactose, but also by
ariety of β-galactosides. One of these,
MG) in which the glucose moiety of
ose is replaced by a thiomethyl group
a convenient inducer since it is not
tabolized by the cells. The structure of
inducer is compared with lactose in
ure 5. When inducer (in sufficient con-
trations) is added to exponentially
wing cells enzyme, synthesis begins
mediately and reaches a maximum
ue after about 4 hours (fig. 6). If the
a are plotted as a function of the in-
ase in protein or dry weight (fig. 7),
obtain the differential rate of synthesis.
is simply tells us that β-galactosidase is
ng made as a constant fraction of newly
thesized protein.

ur first approach to the effect of UV on
oli was to expose cells to varying doses
of UV, then place them in growth medium with inducer and $S^{35}O_4$ in order to measure new protein synthesis. Figure 8 illustrates some typical results. Cell survival is most sensitive followed by induction of β-galactosidase, whereas total protein synthesis

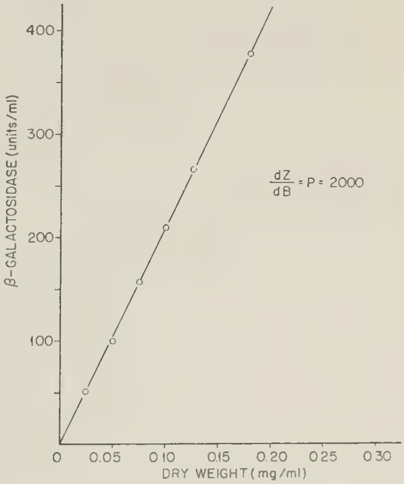


Fig. 7 Differential rate of β-galactosidase synthesis.

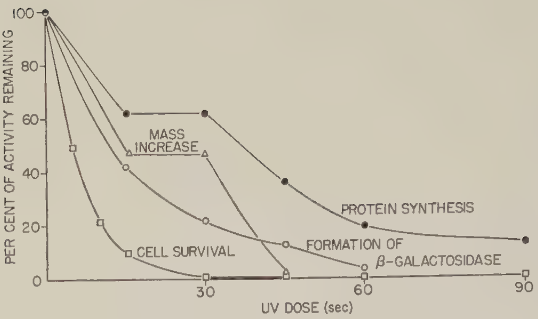


Fig. 8 UV-dose effect on various activities of *E. coli* B grown in 1% succinate.

was the most resistant parameter measured. Figure 9 illustrates the UV dose effect on β-galactosidase synthesis when cell are grown on two different carbon sources The reason for the greater sensitivity o enzyme synthesis in cells grown on glycerol is not readily apparent, but may b related to glycerol metabolism. Since variety of properties sensitive to UV damage can be partially or totally restored b photoreactivation (Kelner, '53; Jagger, '58 Rupert, '61), an attempt was made t photoreactivate induced enzyme synthesis The data of figure 10 show that the in

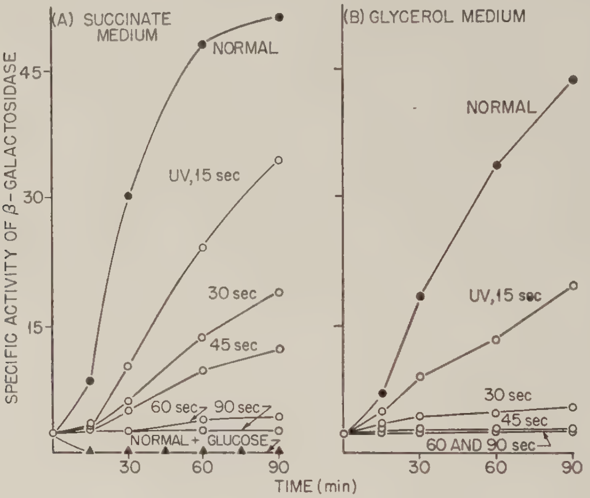


Fig. 9 UV-dose effect on induced synthesis of β-galactosidase.

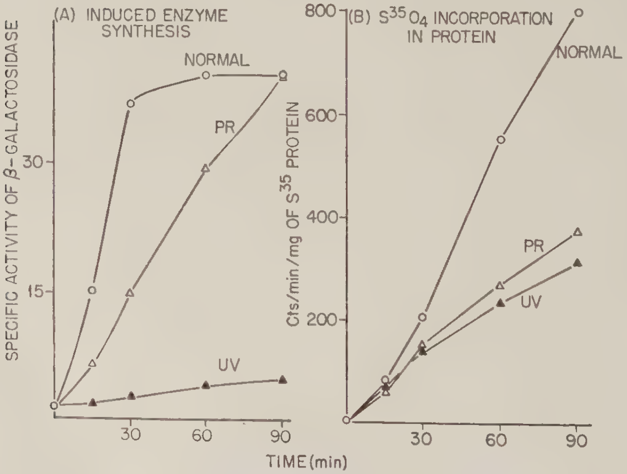


Fig. 10 Differential effect of photoreactivation on induced enzyme synthesis.

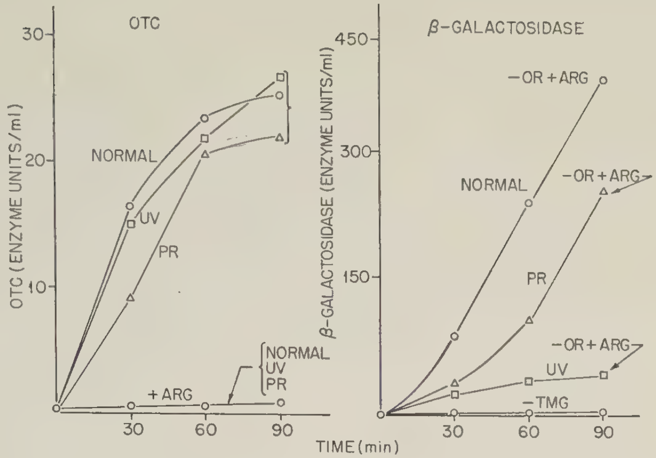


Fig. 11 Simultaneous biosynthesis of OTC and β-galactosidase by normal, UV and PR *E. coli* B.

ed synthesis of β-galactosidase is essen-
y restored to normal by exposure of
to white light after UV irradiation.
restoration of enzyme synthesis seems
e preferential, since total protein syn-
is as measured by $S^{35}O_4$ incorporation
not restored to normal. Thus the dif-
ntial rate of synthesis (dz/db) is in-
sed in favor of β-galactosidase. This
rvation presented us another oppor-
ty to test the idea proposed earlier
rding the difference between β-galacto-
se and OTC. Figure 11 gives a com-
son of the effect of UV and PR on the
hesis of these two enzymes. Again
e is a striking difference between the
systems. OTC synthesis is resistant
JV whereas β-galactosidase synthesis
ensitive. These observations are also
sistent with the hypothesis that β-galac-
dase synthesis requires synthesis of
A although OTC synthesis does not.

aving observed the inhibition of β-
ctosidase induction by UV and its
oreactivation, we were now ready to
mpt to correlate these findings with
e reported by Kameyama and Suzuki,
Normal, UV, and UV and PR cells
e exposed to $S^{35}O_4$ and simultaneously
ıced to make β-galactosidase. After 30
utes incubation the cells were broken
n and the crude extracts were electro-
resed on Geon. The results are shown
gure 12. Again one can see a striking
of a ribonucleoprotein peak in UV cells
that migrates at about 17 cm from the origin. This peak, however, is not restored by photoreactivation, although enzyme synthesis is nearly normal. The most surprising observation, however, was the finding of a peak of β-galactosidase that did not migrate, but remained at the origin. This fact is illustrated more clearly in figure 13 where only the β-galactosidase activity is plotted. It can be seen that in the normal cells the non-migrating fraction is present; it is absent in the UV-treated cells and is restored by PR. It was possible that the fraction of β-galactosidase remaining at the origin was an artifact and represented a contamination with soluble enzyme. To test this idea purified enzyme was added to an extract of uninduced cells and the preparation was electrophoresed as before. These results are shown in figure 14. It can be seen that the purified enzyme migrated to about 15 cm from the origin. When this enzyme was added to an extract of uninduced cells it again migrated to the same position and a non-migratory component was not visible. Thus it seems clear that the fraction of β-galactosidase remaining at the origin was indeed real and not an artifact of experimental manipulation. By centrifugal analysis it was shown that this fraction of β-galactosidase was ribosomal-bound and in various experiments represented from 1–10% of the total enzyme. At about the same time, Roberts and his co-workers ('59); Cowie

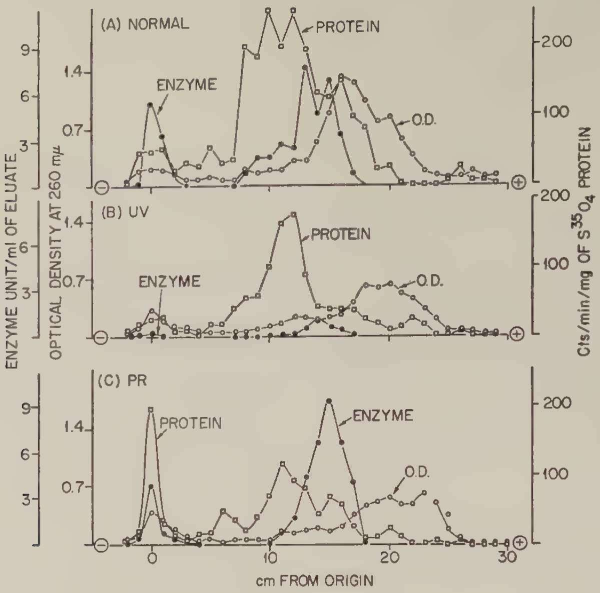


Fig. 12 Geon fractionation of normal, UV and PR cell extracts.

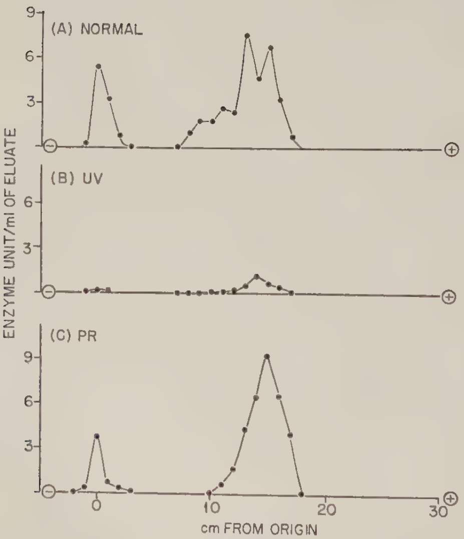


Fig. 13 Geon fractionation of β-galactosidase activity of normal, UV and PR cell extracts.

et al. ('61) reported the observation of a ribosomal-bound fraction of β-galactosidase. These reports represented the first observations of a ribosomal-bound component of β-galactosidase and caused us to speculate on its significance. Since it was well known that removal of inducer led to an abrupt cessation of further enzyme synthesis, we wondered what would happen to the particle-bound enzyme under such conditions. Figure 15 illustrates the course of events after removal of inducer. At zero time inducer was added to the culture and enzyme synthesis occurred as indicated. After 60 minutes of induction the cells were removed from the medium and washed free of inducer. The cells were then re-incubated in the same medium in the presence and absence of inducer for an additional 60 minutes. In the absence of inducer there was no further increase in enzyme content, whereas the cells incubated in the presence of inducer continued to make enzyme at the previous rate. Cells were harvested at the points marked A, B, and C in figure 15, broken open and the crude extracts were subjected to electrophoresis as before. The results of this experiment are shown in figure 16. It is evident that after the initial 60-minute incubation in the presence of inducer, both the particle-bound enzyme and the soluble enzyme are present. An additional period of incubation in the absence of inducer resulted in a loss of the particle-bound component that was added to the soluble pool. Incubation for an additional 60 minutes in the presence of inducer resulted in a further increase of

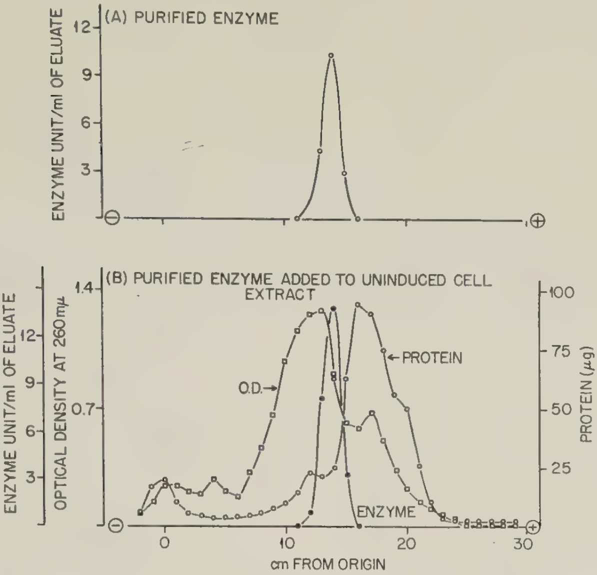
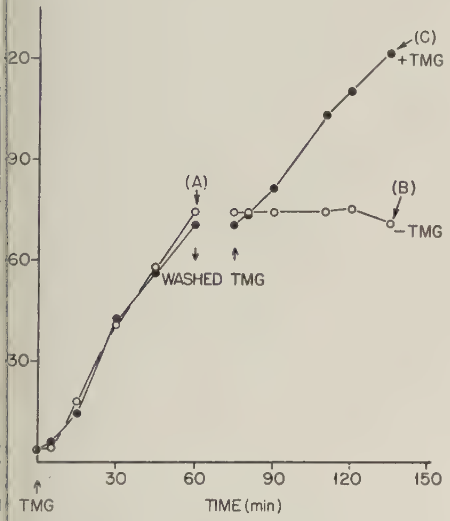


Fig. 14 Geon electrophoresis of β-galactosidase.



. 15 Effect of removal of inducer on β-tosidase synthesis.

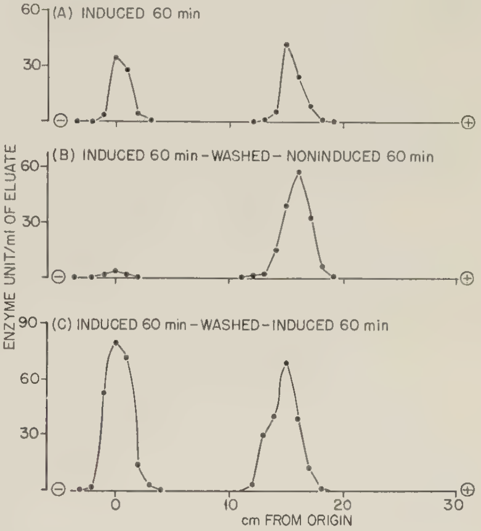


Fig. 16 Effect of removal of inducer on particle-bound enzyme.

components. Since this experiment carried out with UV and PR cells we rpret the excessive accumulation of particle-bound enzyme as a more effecrepair by PR of the synthetic machinhan the release mechanisms. It thus ns evident that the particle-bound form e enzyme can exist only so long as inr is present in the system. This fact ns to be general for induced enzymes, since the same observation has been made with induced sucrose phosphorylase and galactose dehydrogenase in *Pseudomonas saccharophila*, that is a particle-bound form of these enzymes exists as long as inducer is present. Removal of inducer followed by a subsequent incubation results in a disappearance of the particle-bound enzyme (Eisenstadt and Novelli, unpublished observations). If one as-

TABLE 3

Method for preparing cell-free system for synthesis of β-galactosidase

Step
1. 50 Liters log phase *E. coli* cells, pre-induced with TMG for 30 minutes.
2. Cells rapidly chilled, harvested, washed and resuspended in 500 ml Tris pH 7.2, 0.01 M Mg^{++}
3. Suspension passed through French Pressure Cell at 10,000 P_{si}
4. Centrifuged 8,000 × *g* 60′ pellet discarded
5. Centrifuged 30,000 × *g* 90′ pellet discarded
6. Centrifuged 105,000 × *g* 30′ pellet saved = P_{30}
7. Centrifuged 105,000 × *g* 90′ pellet saved = P_{90}
8. P_{30} and P_{90} washed with Tris pH 7.2—0.005 M Mg^{++} collected by centrifugation at 105,000 × *g* 90′
9. Supernatant from step 7, dialyzed vs Tris pH 7.2—0.005 M Mg^{++} for 7 hours = S_{90}

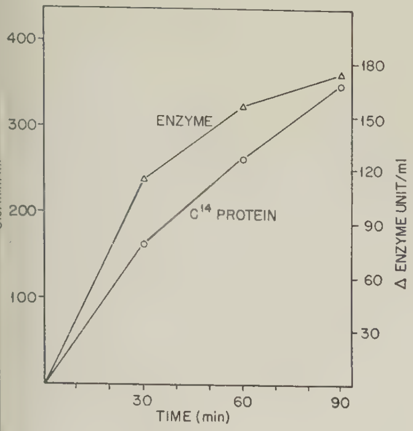
sumes that the particle-bound enzyme represents template-bound enzyme, then one of the older theories regarding the role of the inducer is no longer tenable. This theory stated that the inducer acted by combining with template-bound enzyme and removing it from the specific surface thereby allowing the template to act catalytically (Monod, '58). The present observations demonstrate that particle-bound enzyme can leave and join the soluble pool in the *absence* of inducer.

Cell-free synthesis of β-galactosidase. If the particle-bound β-galactosidase really represents template-bound enzyme, then this particle should participate in enzyme synthesis. Accordingly we set out to make use of this particle in an attempt to develop a cell-free system that would synthesize this enzyme. The method of preparation of the system is given in table 3. When particles and supernatant are combined and supplemented with inducer, amino acids, a source of energy and the nucleoside di- and triphosphates, a 1½ fold increase in enzyme activity is seen upon incubation at 37° for 60 minutes. Typical data are given in table 4. It is evident that the increase in enzyme activity is absolutely dependent upon the presence of inducer and a source of energy. The activity is stimulated by amino acids, by the nucleoside di- and triphosphates and is inhibited by chloramphenicol, ribonuclease, and surprisingly by deoxyribonuclease. The requirements and the effect of inhibitors on the increase in enzyme activity certainly suggests that *de novo* enzyme synthesis is occurring in this system. Several other investigators using various preparations from broken bacterial cells or protoplasts have reported simila increases in enzyme activity when th preparations were incubated with variou supplements (Gale, '55; Spiegelman, '57 Nisman and Fukuhara, '59). It should b pointed out that all of the experiments, in cluding our own, were simply measure ments of an increase in enzyme activit and not of increases in enzyme protein The assumption in all these cases that pro tein synthesis has occurred is inferre from a sensitivity to chloramphenicol, requirement for an energy source, or a re quirement for amino acids. We therefor sought to obtain more direct proof that th observed increase in enzyme activity wa accompanied by an increase in enzym protein. For this purpose the experimen described in table 4 was repeated with th inclusion of C^{14}-leucine in the reactio mixture. At various times samples wer taken and enzyme activity was measured In another sample β-galactosidase was pre cipitated with antisera prepared agains highly purified enzyme and after carefu

TABLE 4

Cell-free synthesis of β-galactosidase

System	Enzyme units per ml		
	Time (minutes)		Net
	0	60	
Complete	145.5	350.5	215
$-S_{90}$	72.5	195.5	123
−TMG	145.5	145.5	0
−ATP and generator	145.5	145.5	0
−AA	145.5	220.5	75
−XTP, XDP	145.5	236.5	91
+CAP	145.5	145.5	0
+RNase	145.5	88.5	−57
+DNase	145.5	93.0	−52.5



17 C^{14}-leucine incorporation into Ab-ppt
n and enzyme formation.

ing of the antigen-antibody precipi-
the radioactivity was measured. Fig-
17 demonstrates that the rise in
me activity was paralleled by a corre-
ding rise in radioactivity of the anti-
ntibody precipitate indicating that
ncrease in enzyme activity is a con-
nce of the synthesis of new enzyme
cules from the free amino acid pool.
5 presents similar data illustrating
equirements of the system. In this
at the end of the incubation period
ncrease in enzyme activity was meas-
the radioactivity of total protein and
tibody precipitable protein was deter-
d. With respect to enzyme activity,
clearly evident that enzyme synthesis
solutely dependent upon particles,
natant, inducer, a source of energy,
the nucleoside triphosphates. These
rements are similarly reflected in the
activity of the antibody precipitates.
nt worthy of note is the presence of
er resulted in an increase of approxi-
ly 1000 cpm in total protein and these
cpm were removed by antibody (com-
lines 1 and 2). In table 6 similar
are presented for a series of inhib-
Again note the effect of deoxyribo-
ase on the system, a marked inhibi-
of enzyme synthesis matched by a
g reduction in incorporation of amino
Glucose is a well-known inhibitor
e induced synthesis of this enzyme
ole cells and it is certainly inhibitory
cell-free preparation. Whether this
is a direct effect of glucose acting as a repressor or to an indirect effect through the formation of acid is not known at this time. The amount of radioactive protein precipitated by antibody in the absence of enzyme synthesis is somewhat disturbing but could be attributable to co-precipitation of some radioactive non-β-galactosidase protein that may have been trapped in the antigen-antibody precipitate. In an at-

TABLE 5

Cell-free synthesis of β-galactosidase and incorporation of C^{14}-leucine: requirements

System	Δ Enzyme units/ml	cpm/ml Total protein	Ab ppt protein
Complete	+183.6	2560	1428
−TMG	− 2.4	1580	384
−AA	+108.0	1276	436
−ATP and generator	− 15.6	182	128
−XTP, XDP	− 24.0	660	248

TABLE 6

Cell-free synthesis of β-galactosidase and incorporation of C^{14}-leucine: inhibitors

System	Δ Enzyme units/ml	cpm/ml Total protein	Ab ppt protein
Complete	+168	2400	1264
+RNase	− 25.8	88	100
+DNase	− 35.4	320	208
+CAP	− 29.4	676	344
+Glucose	− 20.4	808	368

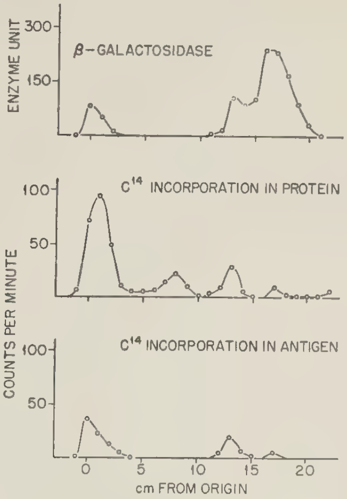


Fig. 18 Geon fractionation of reaction mixture.

tempt to eliminate this difficulty the reaction mixture was precipitated with ammonium sulfate, extensively dialyzed and then electrophoresed on Geon as before. The results of this experiment are shown in figure 18. In the top section of the diagram is shown the distribution of enzyme activity. The middle section shows the distribution of radioactive protein. In order to obtain the data for the bottom section carrier β-galactosidase was added to each tube in order to insure an antigen-antibody precipitate. It should be noted that a radioactive precipitate occurred only in the regions of the electrophoretic diagram that had β-galactosidase activity. The large peak of radioactive protein at the origin, presumably containing ribosomal-bound protein, has radioactive protein other than β-galactosidase, since the antiserum removed only about 40% of the radioactivity. These radioactive ribosomes may be involved in the synthesis of the radioactive protein that occur between 5–10 cm from the origin. It is also apparent from this experiment that the major fraction of the newly synthesized β-galactosidase remains particle bound. This is similar to other ribosomal systems that have been studied for amino acid incorporation (Zamecnik, '60; Mans and Novelli, '61).

As indicated in table 3, the cells were pre-induced before making the cell-free preparation and inducer was again required to be present in the reaction mixture. It was of interest to determine whether *both* particles and supernatant from pre-induced cells are needed to obtain enzyme synthesis. An experiment was performed with combinations of particles and supernatant from non-induced and pre-induced cells with the results illustrated in table 7. It is clearly evident that both components must come from pre-induced cells in order to observe enzyme synthesis. The non-induced system $P_{ni} + S_{ni}$ is able to incorporate leucine C^{14} into protein but is unable to synthesize β-galactosidase in spite of the fact that inducer was present in the reaction mixture.

The requirement for genetically specific DNA in the cell-free synthesis of β-galactosidase. The effect of deoxyribonuclease in inhibiting enzyme synthesis in this system was suggestive of a role for DNA in
enzyme synthesis. However, Allfrey a
Mirsky ('58) had observed a similar i
hibition by deoxyribonuclease of nucle
phosphorylation that could be overco
by DNA, RNA or several unrelated pol
anions. It was therefore necessary to t
to establish a direct effect of DNA in th
system. Kameyama, working in his lab
ratory in Japan, decided to destroy t
DNA by irradiating the supernatant wi
UV and testing the effect of added DN
Such an experiment is illustrated in tab
8. In this experiment particles from t
constitutive strain (P_c) were used wi
supernatant from the induced cells (S
It is clear that treating the supernata
with UV destroys the ability of the syste
to synthesize enzyme, but the addition
DNA from the constitutive strain brin
about a partial restoration of activity. T
DNA itself is also destroyed by UV trea
ment. This experiment also suggests a ro
for DNA in enzyme synthesis, but is n
sufficient to establish the specificity f
DNA.

X irradiation of cells would be a go
way to inactivate DNA, but we had bee
inhibited from X irradiating our syste
because of reports in the literature indica
ing that induced enzyme synthesis is e
tremely resistant to radiation. Thus Bar
et al. ('53) had reported that the induc
synthesis of galactozymase and maltoz

TABLE 7

Comparison of particles and supernatant fr
non-induced and pre-induced cells in the
synthesis of β-galactosidase

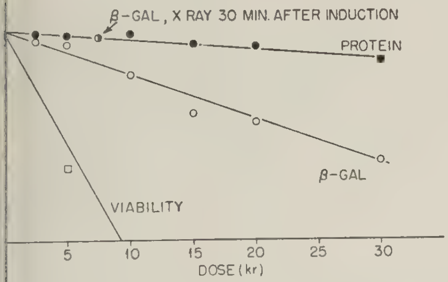
Additions	Δ Enzyme units/ml	cpm/ml Total protein	cpm/m Ab ppt protei
$P_{TMG} + S_{TMG}$	184	2560	1428
$P_{ni} + S_{ni}$	0	2200	48
$P_{ni} + S_{TMG}$	0	2524	0
$P_{TMG} + S_{ni}$	2.8	1812	296

TABLE 8

Effect of UV of supernatant and DNA on
enzyme synthesis

Additions	Δ Enzyme unit 60 min./ml
$P_c + S_i$	575
$P_c + UV\ S_i$	−83
$P_c + UV\ S_i + DNA_c$	124
$P_c + UV\ S_i + UV\ DNA_c$	0
$P_c + none + DNA_c$	0

in yeast was resistant to 300,000 r
hantrenne ('58) found induced cata-
synthesis in yeast to be equally re-
nt. However, Clayton and Adler ('60)
nnection with experiments designed
ermine the effect of peroxide on sen-
y to radiation found that induced
esis of catalase in *Rhodopseudomo-*
pheroides was as sensitive as cell
val to X irradiation. These findings
lated us to test the effect of X irradi-
on the induced synthesis of β-galac-
se in *E. coli.* The results of such an
iment are shown in figure 19. It can
en that although more resistant than
urvival the synthesis of β-galactosi-
is rather sensitive to radiation in the
n of 10,000–40,000 r. We next at-
ed to prepare the cell-free system
X-irradiated cells and found it to be
letely inactive with respect to enzyme
esis. By carrying out mixed experi-
s with particles and supernatants
normal and X-irradiated cells (table
e established that the particles from
diated cells had almost the full ca-
y for enzyme synthesis, but a compo-
in the supernatant had been dam-
by radiation. A nucleic acid fraction
repared from normal supernatant by
omycin precipitation. After removal
eptomycin by dialysis the nucleic acid was tested for ability to restore activity to the cell-free system prepared from X-irradiated cells. The results of this experiment are shown in table 10. The nucleic acid fraction was indeed capable of restoring activity to the damaged system. The active component of the nucleic acid fraction seems to be DNA, since it was sensitive to deoxyribonuclease but not to ribonuclease. That the effect of DNA is specific is evident from the fact that a similar nucleic acid preparation from *Pseudomonas saccharophila*, an organism that does not make β-galactosidase, was inactive in this system. Table 11 further illustrates the specificity of DNA in restoring enzyme synthesis to the X-ray-damaged system. Here only the DNA from the constitutive mutant of *E. coli* is active. DNA's prepared from calf thymus, rat liver, rat spleen, and mouse liver were all inactive. In this case and in all subsequent cases the DNA's are prepared by treating cells with detergent, alcohol precipitated, salt extracted, treated with phenol and precipitated with alcohol. To test whether the DNA effect is gene specific, DNA's were prepared from 4 types of *E. coli* cells. The cells used were the inducible strain (i^+, z^+, y^+) both non-induced (DNA_{ni}) and pre-induced (DNA_i), the constitutive strain (i^-, z^+, y^+) (DNA_c) and a lactose negative strain (i^+, z^-, y^+) (DNA_{lac^-}). The ability



19 Effect of X irradiation on induced syn-
of β-galactosidase.

TABLE 9

arison of damage in particles and super-
natant derived from X-irradiated cells

Additions	Δ Enzyme units/ml
$P_n + S_n$	156
$P_x + S_x$	5
$P_n + S_x$	26
$P_x + S_n$	142

TABLE 10

Effect of added nucleic acid on enzyme synthesis in X ray-inactivated preparation

Additions	Δ Enzyme units/ 60 min./ml
$P_x + S_x$	0
$P_x + S_x +$ nucleic acid (N.A.)	168
$P_x + S_x +$ RNase treated N.A.	177.5
$P_x + S_x +$ DNase treated N.A.	−3.0
$P_x + S_x +$ *P. sacch.* N.A.	−4.5

TABLE 11

Comparison of the effect of various native DNA's on the X ray-damaged system

Additions	Δ Enzyme units/ 90 min./ml
$P_x + S_x$	0
$P_x + S_x + DNA_c$	159
$P_x + S_x +$ DNA (calf thymus)	−12
$P_x + S_x +$ DNA (rat liver)	3
$P_x + S_x +$ DNA (rat spleen)	−4
$P_x + S_x +$ DNA (mouse liver)	0

of these DNA's to restore enzyme synthesis to the X-ray-inactivated preparation was tested and these results are shown in table 12. DNA_{ni}, from the non-induced but inducible strain is inactive, whereas DNA_i from the same strain but where the cells had been pre-induced is fully active. DNA from a mutant that apparently lacks only the gene (z^+) that determines the synthesis of β-galactosidase is inactive. DNA_c from the constitutive strain is also very active. DNA_i, H + C refers to active DNA from induced cells that had been heated to 100° and rapidly cooled, indicating that single-stranded DNA cannot function in this system. These series of experiments establish the fact that a gene which controls the synthesis of a specific enzyme is capable of expressing itself in a cell-free system without requiring that the DNA be replicated. A suggestion regarding the manner by which the genetic information is expressed can be deduced from the experiment shown in table 13. Here it can be seen that DNA cannot function unless the ribonucleotide di- and triphosphates are present. From the experiments of Volkin and Astrachan ('56) and Volkin ('61) it is known that during phage infection and also in normal cells a species of RNA can be found that has a base composition reflecting that of phage DNA (in the case of phage infection) and of normal DNA in normal cells. This species RNA turns over rapidly. In this connect Hurwitz ('60) and Weiss ('60) have tained an RNA polymerase that uses D as a primer and ribonucleoside triph phates as substrates for the synthesis RNA and this RNA reflects the base co position of the DNA used as primer. W these considerations in mind, the exp ment described in table 13 can be int preted by assuming that the information the β-galactosidase gene is transmit through the synthesis of a specific R called messenger RNA that contains information determining the amino a sequence that is specific for β-galacto dase. The operation of this enzyme s thesizing system can be summarized the scheme shown in figure 20. I m point out that this scheme is only ten tive and is used for purposes of discussi It is subject to change as new informati accumulates.

TABLE 12

Effect of various DNA's on enzyme synthesis in the X ray-inactivated preparation

Additions	Δ Enzyme units/ 60 min./ml
$P_x + S_x$	5
$P_x + S_x + DNA_{ni}$	15
$P_x + S_x + DNA_i$	119
$P_x + S_x + DNA_{lac^-}$	−2.5
$P_x + S_x + DNA_c$	170
$P_x + S_x + DNA_i$, H+C	−12.5

TABLE 13

Requirement for nucleoside di- and triphosphates for the DNA-dependent synthesis of β-galactosidase

Additions	Δ Enzyme units/ 90 min./ml
$P_x + S_x$	0
$P_x + S_x + DNA_c$	159
$P_x + S_x + DNA_c$ − XTP, XDP	8

If we summarize the pertinent findin of this investigation, they can be discuss with this scheme as a background. T findings are as follows:

1. In whole cells, addition of induc causes the synthesis of β-galactosidase occur on a ribosome.

2. The ribosomal bound enzyme can released to the soluble pool in the absen of inducer.

3. A cell-free enzyme-synthesizing s tem can be prepared from pre-induc cells, but not from un-induced cells.

4. The cell-free system is composed a particle and a supernatant *both* of whi must be prepared from pre-induced cel

5. The cell-free system requires D from cells that have the gene for β-gala tosidase. If the DNA is prepared from t inducible strain, the cells must be p induced. DNA from uninduced (but ducible) cells is markedly inhibitory (N man and Fukuhara, '60; Finnerty a Novelli, unpublished observations).

(a.) The cell-free system requires t presence of inducer and a full compleme of amino acids. If the system is prepar from the constitutive strain, inducer is r required for enzyme synthesis.

6. In order for DNA to be effective t ribonucleoside di- and triphosphates a required.

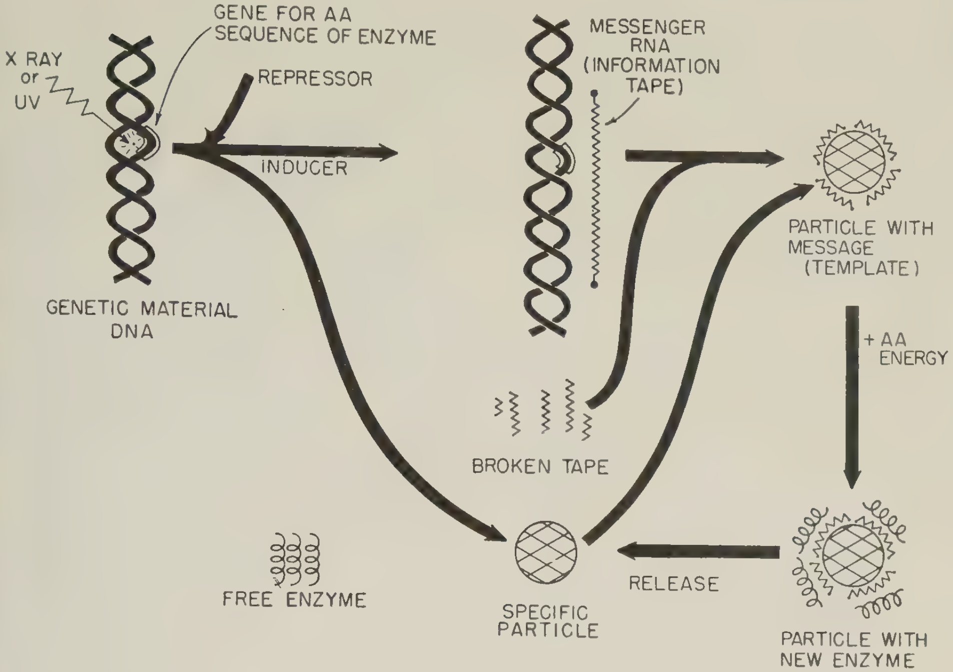


Fig. 20 Scheme of enzyme formation.

Since the cells have the necessary information for making β-galactosidase, but do not do so unless inducer is added, we assume that the z^+ gene is repressed by an unknown product of the i^+ gene. When inducer is admitted to the system it acts by suppression of the i^+ gene and thus z^+ gene is de-repressed and allowed to express itself. This expression is manifested by the synthesis of specific ribosomes and a specific messenger RNA that associates itself with the ribosomes. The combination of specific ribosomes and messenger RNA becomes the enzyme-forming template. Enzyme is synthesized on the surface of messenger RNA and in order for the enzyme to be released to the soluble pool the messenger RNA is destroyed. That the messenger RNA must be labile is a necessary requirement from the experiments of Riley *et al.* ('60) suggesting the absence of a stable cytoplasmic gene product and a requirement for the continual presence of functional DNA in the synthesis of β-galactosidase in whole cells. The requirement for an inducer dependent synthesis of specific ribosomes is forced upon us by the observation that ribosomes from non-induced cells are inactive in the cell-free system. The requirement for inducer in the cell-free preparation even though the cells are pre-induced may be required to keep the z^+ gene de-repressed and suggests that repressor (i^+ gene product) can be made quickly. This is also evident from the fact that removal of inducer from whole cells causes an abrupt cessation of enzyme synthesis. That the repressor is intimately associated with DNA is inferred from the fact that highly purified DNA from non-induced cells is inhibitory.

This is as far as the present facts permit us to go. We believe that this cell-free system represents a specific bio-assay for an individual gene and that it may now be possible to isolate and characterize a gene. The fact that repressed DNA (i.e., DNA_{ni}) inhibits enzyme synthesis suggests that we may also have a bio-assay for the repressor which we hope to isolate, characterize, and determine the mechanism of its function.

SUMMARY

Ultraviolet irradiation of *E. coli* cells was found to inhibit the induced synthesis

of β-galactosidase and the inhibition was reversed by photoreactivation. Examination of crude extracts of such cells led to the discovery of a particle-bound fraction of β-galactosidase. The particle-bound enzyme occurs *only* in the presence of inducer. When inducer is removed the enzyme leaves the particle and becomes associated with the soluble pool. These observations led to the discovery of a cell-free system that can synthesize the enzyme. The system consists of particles and supernatant, both of which must be prepared from pre-induced cells. The system requires the presence of inducer, a source of energy, amino acids, and the nucleoside di- and triphosphates. Enzyme synthesis is inhibited by chloramphenicol, glucose, ribonuclease and deoxyribonuclease. Ultraviolet treatment of the supernatant yielded an inactive preparation that could be made active by addition of DNA from untreated supernatant. Similarly X irradiation of cells destroyed induced enzyme synthesis and cell-free preparations made from X-irradiated cells were inactive. Such inactive preparations could synthesize enzyme when supplemented with DNA. Only DNA's prepared from cells containing the gene for β-galactosidase are effective.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Carl Wust for help in the preparation of antiserum and for advice in carrying out the immunological experiments. We would like to thank Dr. H. I. Adler for assistance in carrying out the X-irradiation experiment.

OPEN DISCUSSION

VON BORSTEL[2]: Have you brought in microsomes from other sources into the system to see if they would act as repressors?

NOVELLI: When we use the particles from non-induced cells, they don't work. I should add that DNA from other sources has been tested also—that is, DNA from other micro-organisms—rat liver, rat kidney, brain, any that we could get our hands on were tested and none of these were active in the system.

VON BORSTEL: What about big pieces of RNA?

NOVELLI: I haven't added that. We are in the process now of trying to fractionate the system to get these components out to try to fractionate messenger RNA.

ADLER[3]: One of the striking things about induced enzyme synthesis seems to be the similarity between the inducer and the substrate of the enzyme. Either the substrate is the inducer or, when it is not at least something very similar in structure is the inducer. Would you care to comment on how this similarity fits or doesn't fit with the scheme on the last figure?

NOVELLI: I really don't know. I am sure it has some significance because what you say is true. One can say the same thing about the transcarbamylase where we believe that some derivative of arginine shuts down the entire pathway.

Our data seems to indicate that whatever the repressor is it acts at the level of the enzyme-forming machinery. This means that, if there are different particles associated with the synthesis of the different enzymes in that chain in arginine bio-synthesis, those particles have to have some degree of similarity in order that the repressor can recognize these and shut them down.

The alternative is that the repressor acts at the level of the gene. Again there has to be a similarity in structure in order that a single repressor can shut down all these genes. But the data that we have so far is from Dr. Rogers who tested DNase in his cell-free system. He did not obtain as striking an effect of DNase as we do with the β-galactosidase system. He gets about a 50% inhibition. Sometimes he doesn't get any at all. I think this reflects that the ornithine transcarbamylase messenger probably has a longer half-life than that of β-galactosidase.

UPTON[4]: Why do you think the single-stranded DNA is not active? Another question: If the single-stranded DNA is inactive, is the 2-stranded DNA radiosensitive *in vitro*?

---

[2] R. C. von Borstel, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

[3] H. I. Adler, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

[4] A. C. Upton, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

NOVELLI: I don't know why the single- anded DNA is not active; I can just say at it is not. We have not done X-ray nsitivity of DNA *in vitro*.

KELLY[5]: How did you purify the DNA? it possible that you are carrying along small amount of RNA so that you are tually carrying along the messenger NA or do you believe that it is being ade in a cell-free system?

NOVELLI: The DNA was purified rough a detergent and a phenol proce- re. If RNA is being carried at all, it is t sensitive to ribonuclease because we ok the purified DNA, treated it with Nase or DNase, re-isolated it through enol to destroy the added nuclease; and ly the DNase treatment destroyed the tivity. RNase did not cause a loss of tivity. But I should point out that Mar- ur discussed this point with me. He inks that if there is this hybrid RNA- NA mixture, it may not be sensitive to Nase.

MARCOVICH[6]: Did you induce *in vitro* NA with TMG or *in vivo* and then use it ter extraction? If you have a strain hich is non-inducible, could you induce *in vitro*?

NOVELLI: For DNA to be effective in e cell-free system, the cells from which e DNA is to be prepared must come om pre-induced cells, if one is using the ducible strain. But the DNA so prepared not active in the cell-free system unless ducer is present in the reaction mixture. e have not been able to induce DNA *vitro*.

ROGERS[7]: I think it is fine that TMG required for maximum enzyme synthe- when you add constitutive DNA. Since u are using an induced supernatant, $_i$), there is probably plenty of repressor hich would be expected to block β-galac- sidase synthesis in the absence of TMG.

The thing that does surprise me is the ct that there is a difference between non- duced and induced DNA. Both of these NA preparations are from i^+ cells, yet ly DNA from preinduced cells allows galactosidase formation. I would expect th preparations to work the same. In th cases the S_i supernatant (presumably ntaining some repressor molecule) plus MG are present.

The reaction:

$$\text{Repressor} + \text{DNA}_i \underset{+\text{TMG}}{\overset{-\text{TMG}}{\rightleftharpoons}} \text{DNA}_i - \text{repressor}$$

must be freely reversible, since, where TMG is washed out of whole cells, β-galactosidase synthesis stops. Thus I would expect TMG to operate in the same fashion in both cases: shifting the equilibrium to the left; displacing the repressor; and allowing β-galactosidase synthesis.

So the difference you observe when you add back induced versus non-induced i + DNA is unexpected. Another point I would like to make concerns the ornithine transcarbamylase system. In the experiments with the uracil-requiring mutant, there is a small amount of $P^{32}O_4$ incorporation into RNA in the absence of uracil. It may very well be that the formation, breakage, and reformation of the messenger-RNA you have postulated required only a small pool of uridine nucleotides. So this will go along with your idea of the repressor acting at the DNA level.

However, the other explanation, suggested by this experiment with the uracil-requiring mutant, is that here the repressor is acting at a different level. Most likely, in the case of arginine repression of ornithine transcarbamylase, the repressor acts at the particle surface, which excludes the necessity for new RNA synthesis.

Another thing I want to ask you a question about: Have you ever put the induced particles together with the constitutive supernatant? Do you require TMG in that system? In other words, if you use your pre-induced particles, do you require TMG?

From the things we know about or the little we know about the repressor in the system, you would expect the S_i supernatant to have the information to make a repressor, wouldn't you?

So the experiment that should be done here would be to see if TMG is required when constitutive supernatant (S_i) is added to pre-induced particles (P_i). If it is, this will be quite a surprise.

---

[5] Lola S. Kelly, University of California, Berkeley.

[6] Herbert Marcovich, Laboratoire Pasteur, Institut du Radium, Paris.

[7] Palmer Rogers, Ohio State University.

NOVELLI: The kinetic curve I showed was of an X-ray-inactivated system derived from an inducible strain to which was added DNA prepared from the constitutive strain with the presence and absence of inducer. In both cases enzyme synthesis was normal for 30 minutes. Enzyme synthesis continued for an additional 30 minutes when inducer was present, but stopped in the absence of inducer. This observation suggests that the 1^+ character in the supernatant had begun to express itself. We have tested induced particles with constitutive supernatant and observed good synthesis in the absence of inducer, but since the DNA in such a system is i^-, one would not expect an inducer effect. I think the situation is more complex than your mass action equation suggests. The fact is that only derepressed DNA works and it also requires inducer in the reaction mixture. This is a surprise and needs an explanation that will be forthcoming only when more experiments with different combinations of particles, supernatants, DNA's with and without inducer can be carried out.

CLAYTON[8] : It seems established now that there are cases in which the synthesis of a single enzyme is as sensitive to X rays as is the ability of a whole cell to survive. Furthermore, the DNA is the X-ray-sensitive component.

Would Dr. Novelli like to elaborate on theories for the X-ray mechanism that will accommodate these facts?

NOVELLI: I realize that in the case of β-galactosidase X-ray sensitivity of induction is not the same as that of induced catalase in Rhodopseudomonas spheroides. That data wasn't shown, but Clayton and Adler's data indicate that over the entire range of X-ray dose sensitivity of induced catalase follows the same curve as the viability.

I don't have any explanation for this except to say that maybe the gene that regulates catalase lies in the region that is very sensitive with respect to growth.

CLAYTON: The question I had was essentially this, that survival may depend on or require the synthesis of a number of proteins, and so there should be a greater multiplicity or number of targets for that than for the synthesis of one enzyme. This leads one to think in terms of a single X-ray hit inactivating a class of genes or a mechanism for the expression of a class of genes.

NOVELLI: One can argue that, in order for a gene to express itself, it has to be a part of a larger component. The information unit itself may be relatively small but in order to express itself, it has to be part of a large component.

One can make an analogy between the gene and, let's say, the active site of an enzyme. The active site of an enzyme probably involves only about 3 or 4 amino acids. Yet if you destroy the larger structure, the expression of that active site is destroyed, even though the destruction may be at a point far removed from the active site.

It may be that in this particular organism there is an arrangement of the genes so that a hit in some region involving viability is also part of the superstructure necessary for the expression of the catalase gene without requiring that the gene itself suffer a hit.

GRAY[9] : I would like to describe two experiments which I think are relevant to this discussion. They present some published data (X-ray sensitivity and target volume of enzyme induction. Nature, *184:* 1570, 1959) and unpublished observations by Dr. H. Pauly of the Max-Planck-Institut für Biophysik, Frankfurt, on the effect of radiation on the capacity of *Bacillus cadaveris* to synthesize the adaptive enzyme lysine decarboxylase. The first shows that the time taken for enzyme synthesis to reach this peak level is the same in irradiated and unirradiated organisms, but that the maximum yield itself is depressed by irradiation. The second experiment shows two things:

1. That the logarithm of the capacity for enzyme synthesis is linearly related to the dose, indicating that a one-hit mechanism is involved, and 2. That the slope of the broken line (unpublished), which represents the loss of capacity for enzyme synthesis by organisms irradiated in the presence of oxygen, is about 4 times as

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[8] R. K. Clayton, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

[9] L. H. Gray, Mt. Vernon Hospital, Northwood.

eat as that for organisms irradiated aerobically.

These results indicate not only that loss capacity for adaptive enzyme synthesis ay be initiated by a single particle, but so that the target for inhibition of en- yme synthesis is only about a factor of 4 so smaller than that for loss of colony rming ability (when the two effects are mpared for irradiations at the same oxy- n tension).

If I could make one very large extra- olation from these results, it would be suggest that the same approximate uivalence, or near equivalence, in the nsitivity with respect to capacity for daptive enzyme synthesis and for loss colony forming ability may possibly xist in the case of mammalian cells, and should very much like to know whether is is, in fact, the case. I have in mind articularly the effect of radiation in delay- g the onset of DNA synthesis in regen- ating liver—a phenomenon which has en extensively studied by Dr. Kelly and hers. We know that a necessary condi- on for the onset of regeneration is the ap- earance of the adaptive enzyme thymidy- c kinase. If I am right in suggesting this rallel may exist between the effects of radiation on enzyme synthesis in bacteria nd in regenerating liver, I think we may well on the way towards tracking down e phenomenon of the inhibition of DNA nthesis in the living cells, and this in rn might lead us to a better understand- g of the effects of radiation on the syn- esis of both constitutive and adaptive nzymes in other types of mammalian lls.

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